EFFECTS OF POLYMYXINS ON THE CELL ENVELOPE OF ESCHERICHIA COLI

BY

RONALD ANTHONY DIXON

A thesis submitted for the degree of

Doctor of Philosophy

in the University of Bristol

DEDICATION

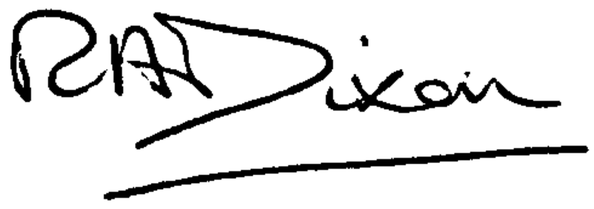
This thesis is dedicated to the memory of my father,

Arthur Dixon (1916-1979)

MEMORANDUM

I declare that this thesis represents my own unaided work except where otherwise acknowledged.

The material has never been presented to the University of Bristol or any other establishment for the purpose of obtaining a higher degree.



R. A. Dixon

Department of Microbiology

University of Bristol

November 1986

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Ian Chopra for his advice, guidance and constant encouragement during both the execution and the reporting of this work.

I thank Professor A. H. Linton for making laboratory space available in the Department of Microbiology; Dr. T. G. B. Howe and Dr. A. J. Hedges for helpful discussions and advice; Mrs. P. Stirling for her photographic expertise; Mr. R. D. Harber for the considerable time and effort spent in introducing me to the complexities of microcomputers and the technical staff of the Department for support services; Miss F. Morton for performing the experiment described in fig 5.3; Mrs. S. Johnson and Mr. K. Hacker for helpful advice and preparing some of the electrophoresis samples. I also thank all my colleagues within the Department especially Dr. M. Shohayeb, Mr. D. Davies and Miss A. Matranga for helpful discussions. Thanks are due to Dr. P. Langford for proof reading this manuscript, any errors which remain are mine alone.

I wish to express my gratitude to Dr. K. R. L. Mansford and the management of Beecham Pharmaceutical Research Division for providing facilities for work carried out at Brockham Park. I thank Dr. J. Heyes and Dr. G. N. Rolinson for their help, especially at the inception stage of this project. I would particularly like to thank my industrial supervisor Mr. M. J. Basker for his help and advice throughout this studentship; Dr. M. Wilkinson for his specialist advice and for providing TEM

photographs; Mr. J. Warrack for providing SEM photographs; Mr. A. Robbins for instruction in the preparation of samples for EM; Mr. S. Box and Mr. S. Spear for supervising the preparation and analysis of PMBN.

I would also like to thank Dr. M. Dunn and his colleagues at Hammersmith Hospital, London for introducing me to new methods in two-dimensional PAGE.

Finally thanks are due to my family for their patience and consideration throughout the last three years and especially to my wife for the preparation of this manuscript and without whose support this work would not have been completed (or started!).

This work was supported by a Science and Engineering Research Council CASE Studentship in collaboration with Beecham Pharmaceutical Research Division.

PUBLICATIONS

Parts of the work presented in this thesis have already been published.

DIXON,R.A.& CHOPRA,I.(1986). Leakage of periplasmic proteins from Escherichia coli mediated by polymyxin B nonapeptide. Antimicrobial Agents and Chemotherapy 29:781-788.

DIXON,R.A.& CHOPRA,I.(1986). Polymyxin B and polymyxin B nonapeptide alter cytoplasmic membrane permeability in Escherichia coli. Journal of Antimicrobial Chemotherapy 18:557-564.

It is proposed to prepare a third manuscript concerning the electron microscopy studies described in Chapter 7.

ABBREVIATIONS:-

CAT_{12}	=	4-dodecyl dimethyl ammonium-1-oxyl -2,2,6,6-tetramethyl piperidine bromide
c.p.m.	=	Counts per minute
dab	=	Diaminobutyric acid
EDTA	=	Ethylenediaminetetraacetic acid
LPS	=	Lipopolysaccharide
MIC	=	Minimum inhibitory concentration
PAGE	=	Polyacrylamide gel electrophoresis
PBP	=	Penicillin binding protein
SDS	=	Sodium dodecyl sulphate
SEM	=	Scanning electron microscope
TEM	=	Transmission electron microscope
TRIS	=	Tris(hydroxymethyl)aminomethane

CONTENTS

SYNOPSIS

The antibiotic polymyxin B acts principally by disruption of cellular membranes in Gram-negative bacteria. Its deacylated derivative polymyxin B nonapeptide (PMBN) lacks antibacterial activity but interacts with the cell envelope and demonstrates synergy with a number of antibiotics to which the Gram-negative cell is not normally susceptible. In order to investigate the mode of action of PMBN, the studies described in this thesis have compared the effects of PMBN and polymyxin B on cell envelope structure and function. Both compounds affected the integrity of the cell envelope of Escherichia coli since they caused release of proteins from cells. The origin of the released protein was determined by polyacrylamide gel electrophoresis and by using specific enzyme markers. The proteins released by both compounds were derived principally from the periplasm indicating that the outer membrane must have been damaged to allow release. Although polymyxin B and PMBN both caused release of periplasmic proteins, the individual proteins released by the compounds differed. Polymyxin B caused considerable release of a number of specific polypeptides. In contrast PMBN released different individual polypeptides. PMBN-treated cells were more susceptible to lysis by lysozyme and accumulated the hydrophobic dye crystal violet, providing further evidence for the loss of outer membrane integrity promoted by PMBN.

The effects of polymyxin B and PMBN on the permeability of the inner membrane of E.coli were also investigated. Both

compounds caused loss of free amino acids, uracil and K^+ from E.coli. The rates of loss promoted by polymyxin B were one and a half to two-fold greater than those caused by PMBN. Loss of low molecular weight substances by polymyxin B may reflect changes in membrane integrity, leading to loss of cytoplasmic proteins and subsequent lysis of the cells. Although PMBN also induced loss of small molecules from E.coli it was not bactericidal.

Electron microscopy studies showed that polymyxin B and PMBN both caused surface damage in E.coli. However, PMBN produced less extensive morphological changes in the cell envelope than polymyxin B.

CHAPTER 1

GENERAL INTRODUCTION

The experimental work described in this thesis has been concerned with the effects of certain membrane-active antibiotics upon the E.coli cell envelope. This general introduction provides a summary of E.coli cell envelope structure and function to complement the more specific aspects detailed in later chapters.

1.1 GENERAL FEATURES OF THE CELL ENVELOPE OF GRAM-NEGATIVE BACTERIA

The cell envelope of a Gram-negative cell is a highly complex structure and has been the subject of a number of recent reviews (Braun & Hantke, 1974; Costerton et al., 1974; Rogers et al., 1980; Lugtenberg & Van Alphen, 1983; Nikaido, 1985 and Nikaido & Vaara, 1985). The cell envelope consists of a three tier structure, the cytoplasmic (inner) membrane, the peptidoglycan (murein) layer and the outer membrane (see fig 1.1). The outer membrane, however, is not always the outermost layer of the cell envelope as it is often covered with an amorphous capsular material consisting of polysaccharide. Surface appendages such as flagella, fimbrae and pili are anchored in the cell envelope (DePamphilis, 1971), but project from the outer layers of the cell into the surrounding environment.

1.1.1 Cytoplasmic membrane

The cytoplasmic membrane of Gram-negative bacteria is composed mainly of lipids (phospholipids) arranged in a unit membrane

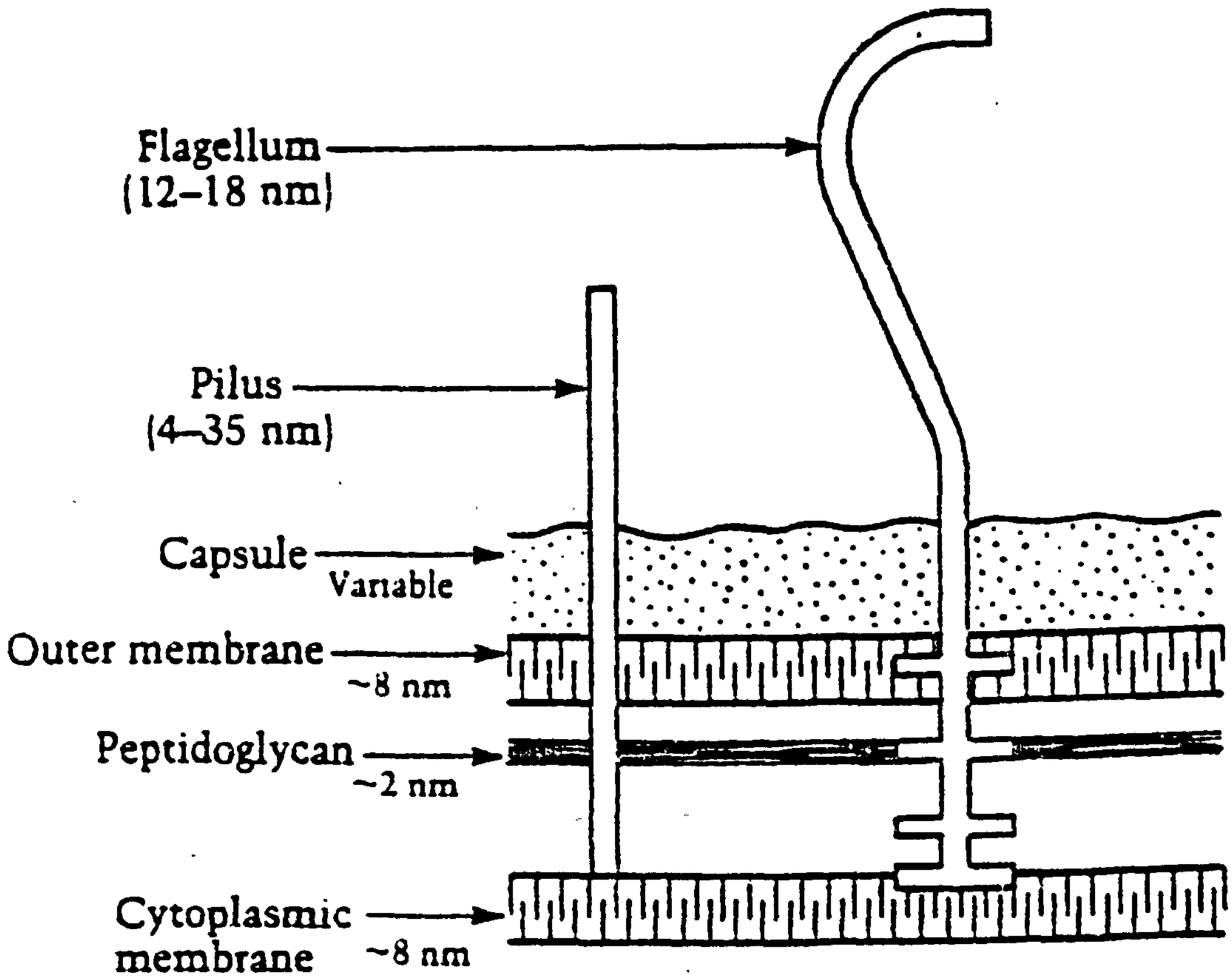


Fig 1.1 The structure of the cell envelope of Gram-negative bacteria showing the major molecular components and their approximate dimensions. (From Ingraham et al 1983).

bilayer structure (Salton & Owen, 1976). Basically the inner membrane provides an osmotic barrier for the cell. The hydrophobicity conferred by the phospholipids is modified by the presence of a wide variety of polypeptides in the membrane which allow access of nutrients. The cell membrane plays a crucial role in macromolecular syntheses with virtually all metabolic reactions of the cell occurring within or internal to the membrane. The major functions of cytoplasmic membrane proteins are energy generation, active and facilitated transport of nutrients, export of by-products and enzymic synthesis of cell envelope components.

1.1.2 Murein (peptidoglycan) layer

In Gram-negative bacteria, a monolayer of peptidoglycan surrounds the cytoplasmic membrane although it is probably not attached covalently to it. The mechanical strength of peptidoglycan reflects its unique structure. It consists of a network of linear amino sugar chains containing alternate residues of N-acetylglucosamine and N-acetylmuramic acid. They are covalently linked to each other via tetrapeptide residues which are attached to the N-acetylmuramic acid (see Schleifer & Kandler, 1972 for review). The peptidoglycan layer provides Gram-negative cells such as E.coli with rigidity and can in addition be partly responsible for the rod shape of the cell. The peptidoglycan in the cell is firmly bound to the outer membrane by covalent linkage of murein lipoprotein to some of the tetrapeptide residues of the peptidoglycan. This attachment is reinforced by non-covalent bonding between the

peptidoglycan and other proteins of the outer membrane.

1.1.3 Outer membrane

The outer membrane of Gram-negative bacteria forms the barrier between the external environment and the cell. It plays a role in receptor-mediated interactions with other cells, phages and colicins. In addition the outer membrane participates in conjugation between bacteria and adhesion receptors of certain bacteria to their mammalian host. (Curtiss, 1981; Gaastra & De Graaf, 1982; Chopra & Linton, 1986 and Chopra, 1986).

The outer membrane contains, in addition to phospholipids and proteins, lipopolysaccharide (LPS) as a major constituent. The current model for its organisation is shown in fig 1.2 (Nikaido & Vaara, 1985). Structurally the membrane is composed of a bilayer of two opposed lipid leaflets. The inner leaflet contains phospholipid molecules identical to those found in the cytoplasmic membrane. However, phospholipid in the outer leaflet appears to be largely replaced by LPS - a molecule unique to the outer membrane of Gram-negative cells. Thus, the membrane exhibits unusual asymmetry. The LPS carries a net negative charge resulting in a strong negative surface charge on Gram-negative cells. The presence of LPS in the outer membrane plays an important role in the impermeability of the outer membrane to hydrophobic compounds (Nikaido, 1976). Recent X-ray diffraction studies (Labischinski et al., 1985) have demonstrated a high state of order in isolated LPS, which may possibly account for its function as a permeability barrier to hydrophobic molecules. Adjacent LPS molecules in the outer

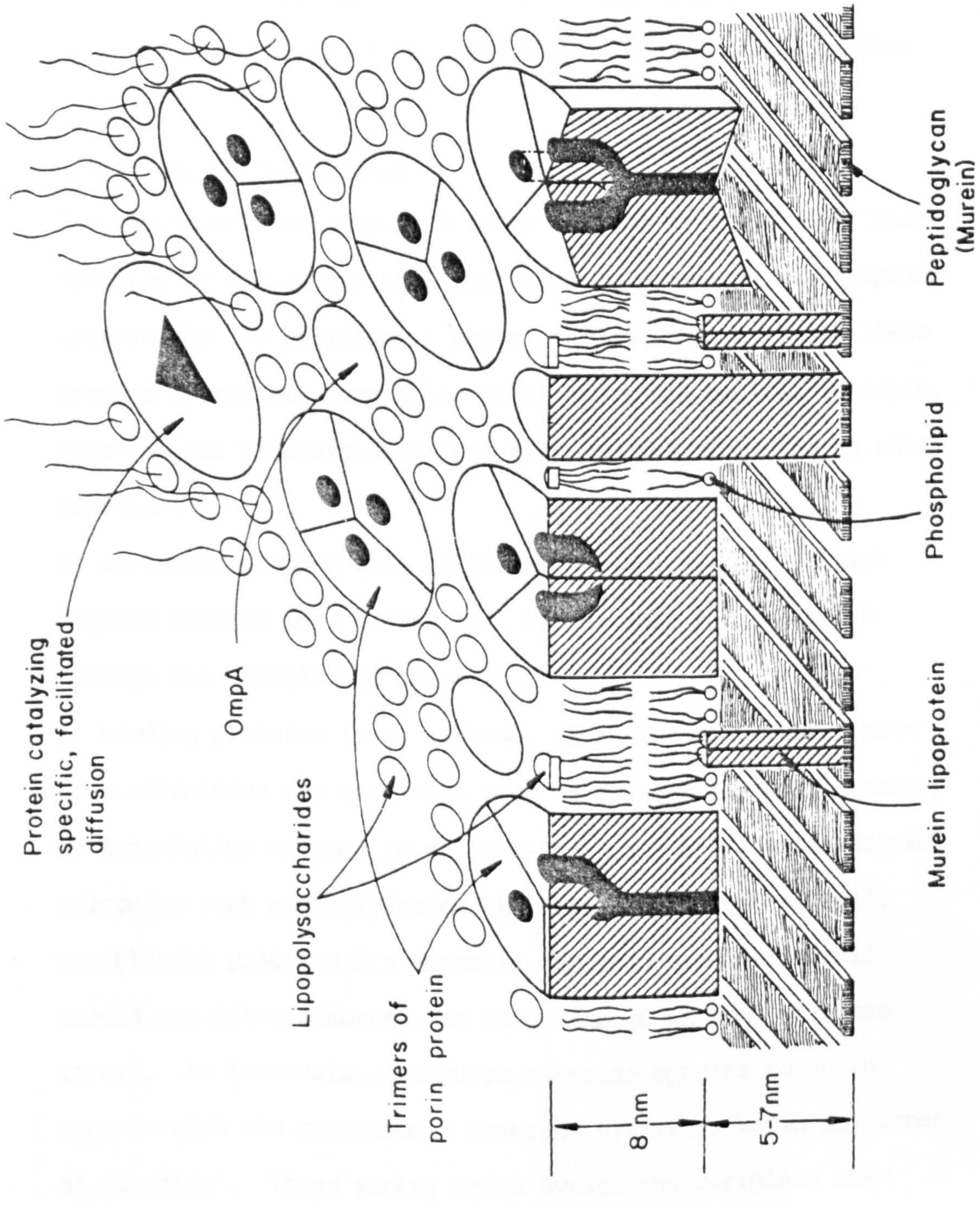


Fig 1.2 Schematic diagram of the outer membrane of E.coli.
(From Nikaido & Vaara 1985).

membrane bilayer are stabilized in part by the presence of divalent cations (see later). The outer membrane is almost devoid of enzymatic activity, only a phospholipase and a protease are known to be located there (Vaara & Viljanen 1983).

1.1.4 Periplasmic region

The region between outer and inner membranes is only partially occupied by peptidoglycan. This area is known as the periplasm (previously the periplasmic space) and contains at least 30-50 species of protein comprising 4% of the total cellular protein. Proteins can be classified into three groups according to their function:

a) catabolic enzymes (e.g. alkaline phosphatase etc.) which degrade solutes into a form that is suitable for transport through the cytoplasmic membrane.

b) binding proteins (e.g. maltose, glutamine etc.) which have high affinities for nutrients such as amino acids and sugars.

c) degradative enzymes (e.g. beta-lactamase etc.) which degrade molecules such as antibiotics that are harmful to the cell.

Periplasmic proteins are normally retained within the cell unless the outer membrane has been damaged in some way (see later). At intervals, the outer membrane appears to be in contact with the cytoplasmic membrane via areas known as 'zones of adhesion'. These zones, which bypass the periplasm have been implicated in the translocation of newly synthesised LPS and outer membrane proteins from the cytoplasmic membrane to the outer membrane (Lugtenberg & Van Alphen, 1983).

1.2 INDIVIDUAL CONSTITUENTS OF THE OUTER MEMBRANE

1.2.1 Phospholipids

All phospholipids of E.coli are located in the cell envelope. Phosphatidylethanolamine is the major species although substantial amounts of phosphatidylglyerol and diphosphatidylglyerol (cardiolipin) are found (fig 1.3). The fatty acid moieties are designated R_1 and R_2 in the figure. Their structures are shown in fig 1.4. The phospholipid composition of the outer membrane is usually very similar to that of the cytoplasmic membrane. Several authors e.g. Osborn et al., (1972) and Lugtenberg & Peters, (1976) have reported that compared with the inner membrane, the outer membrane of E.coli is enriched for phosphatidylethanolamine but contains less of the two other phospholipid types. The abundance of phosphatidylethanolamine, which may represent as much as 90% of the outer membrane phospholipid, is consistent with its ability to form stable bilayers with LPS (see above). Lugtenberg & Peters, (1976) have shown that when present in the outer membrane this species of phospholipid consistently contains more saturated fatty acids than phosphatidylethanolamine in the cytoplasmic membrane. This probably explains why the E.coli outer membrane is enriched for saturated fatty acids (Lugtenberg & Peters, 1976).

1.2.2 Lipopolysaccharide (LPS)

The structure and function of LPS have been extensively reviewed by a number of authors including Nikaido, (1973);

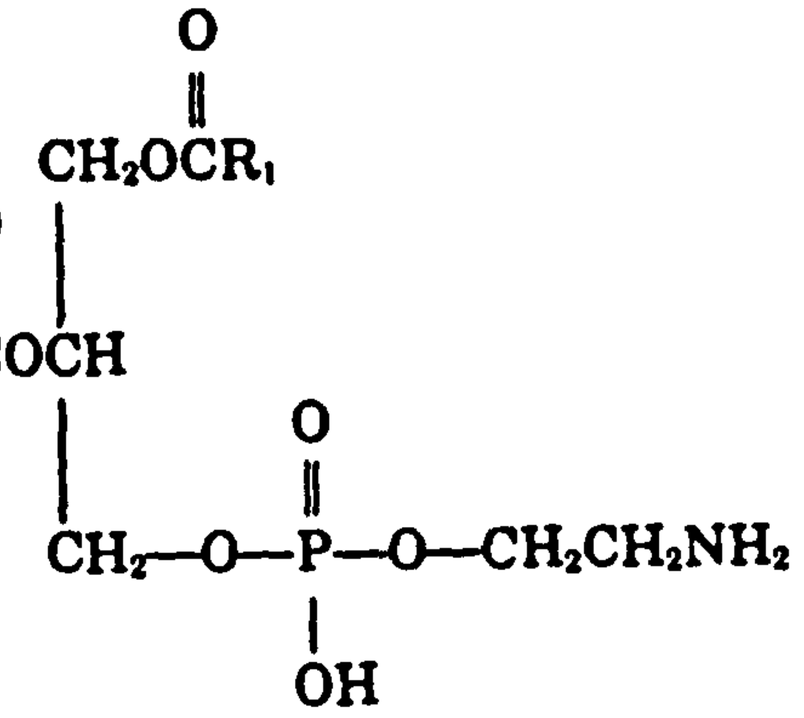
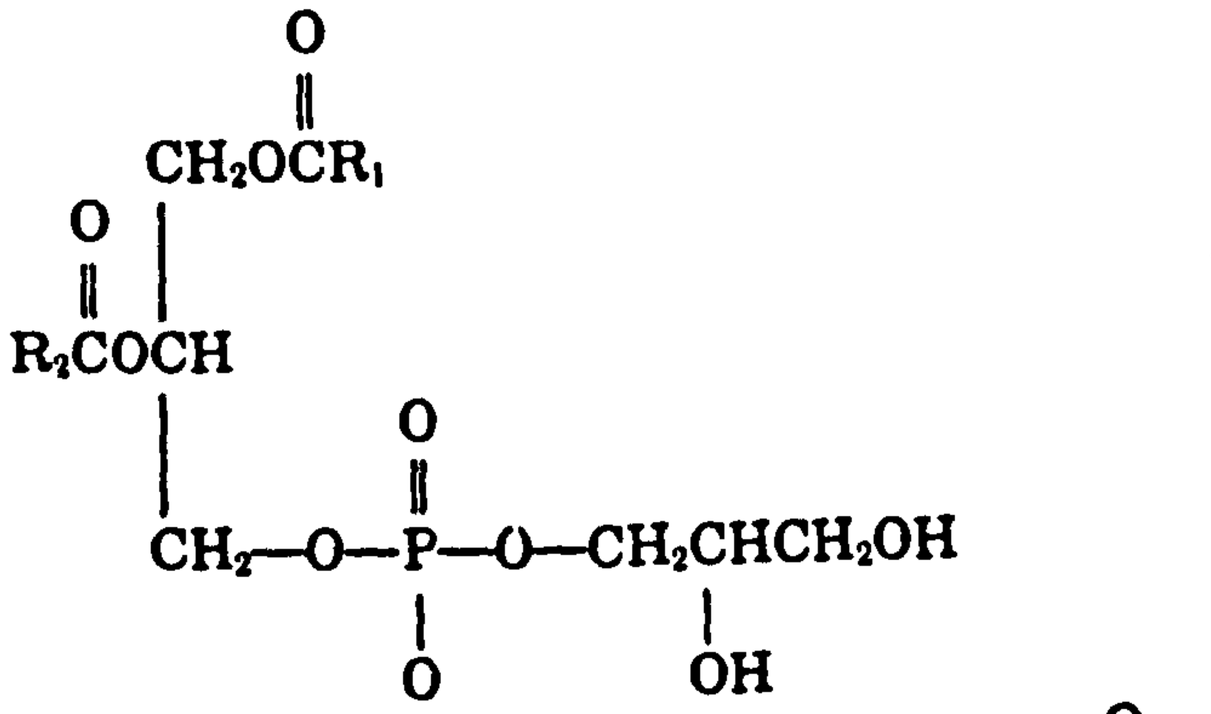
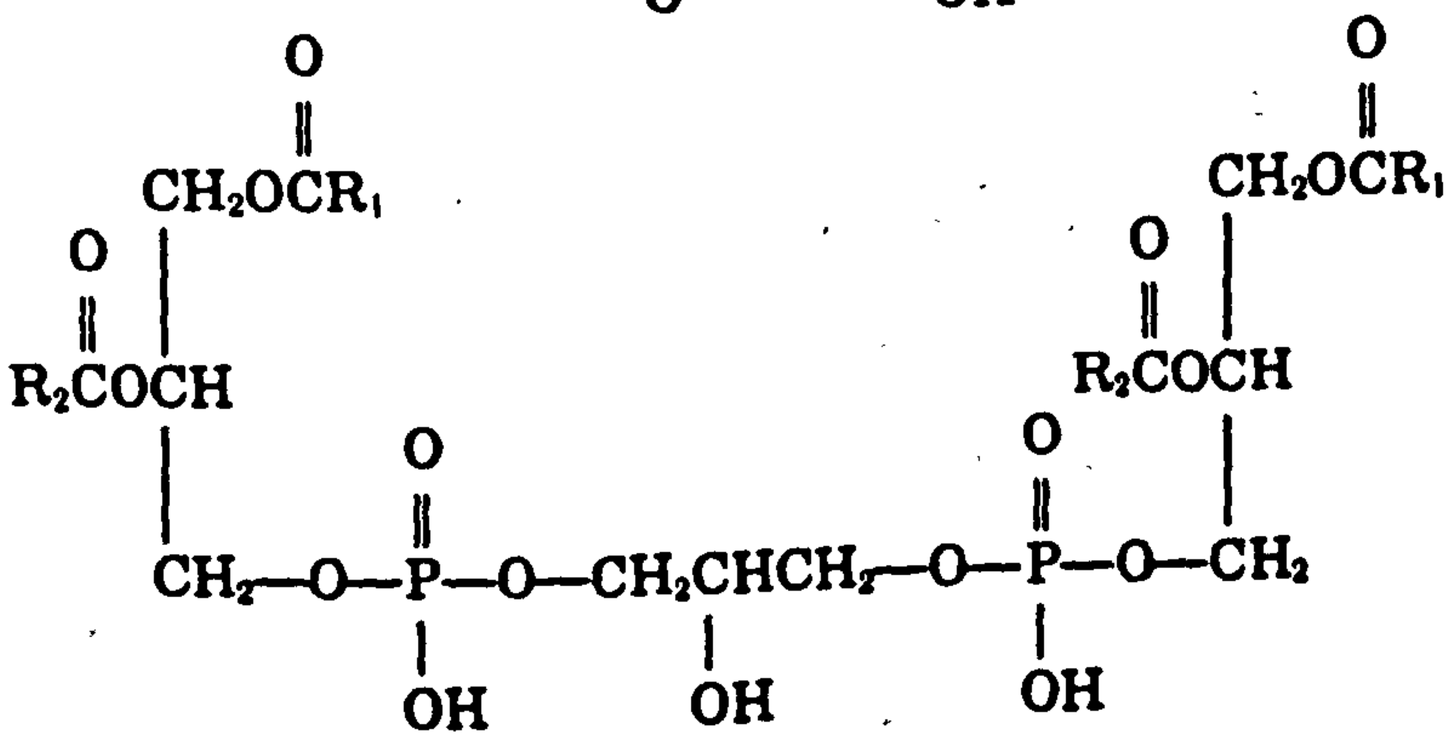
Name	Structure[a]
Phosphatidylethanolamine	
Phosphatidylglycerol	
Cardiolipin	

Fig 1.3 Structure of bacterial phospholipids from E.coli.

R_1 and R_2 = Fatty acyl residues.

Galanos et al., (1977) and Luderitz et al., (1982). The structure of E.coli LPS is shown in fig 1.5. It is an unusual lipidic molecule having a hydrophobic region which contains lipid A. This comprises the lipid moiety of LPS in the outer leaflet. Lipid A is a glycolipid consisting of a glucosamine disaccharide backbone which carries at least two phosphate residues and six or seven fatty acid chains. LPS appears to be anchored in the outer membrane by binding to outer membrane proteins and by non-covalent cross-bridging of adjacent LPS molecules with divalent cations (Leive et al., 1968 and DePamphilis, 1971). The polysaccharide portion of LPS ('R core') tends to be well conserved amongst Gram-negative bacteria and contains the unique sugars, ketodeoxyoctonate (3-deoxy-D-manno-octulosonic acid) (KDO) and L-glycero-D-mannoheptose, both of which are LPS specific. In addition, it contains phosphate, ethanolamine phosphate, and ethanolamine pyrophosphate. Numerous mutants with defects in core structure have been isolated by selection for resistance towards the polymyxins (Vaara et al., 1979). The peripheral part of the core polysaccharide with its attached, often branched, 'O' polysaccharide side chains projects outwards and unlike the 'R' core shows a great evolutionary divergence. The structure of the subunits of the 'O' antigen shows extreme diversity even within a single genus like Escherichia or Salmonella (Orskov et al., 1977). This property is used in serotyping to identify say, substrains of one species in great detail. Colonies of strains with or without the 'O' antigen often have

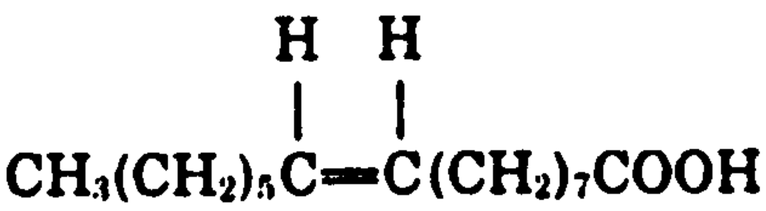
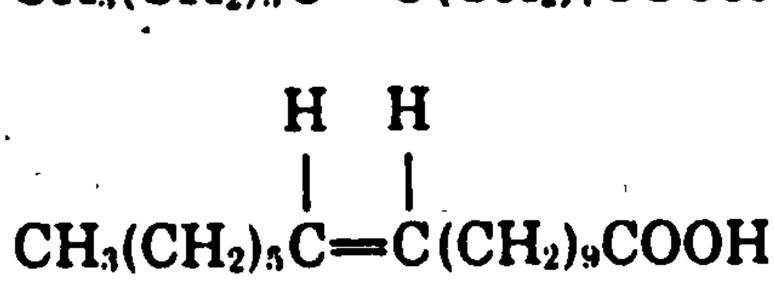
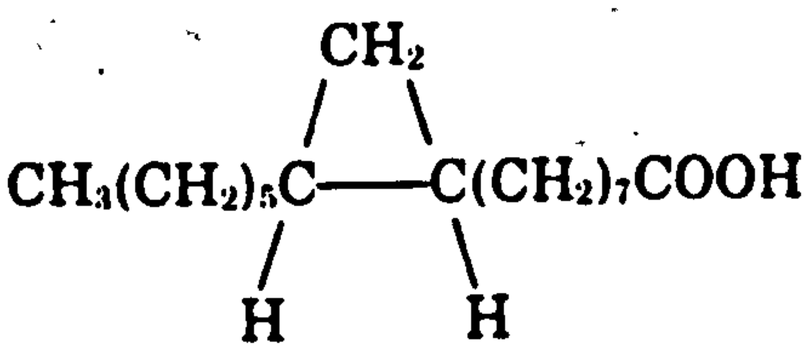
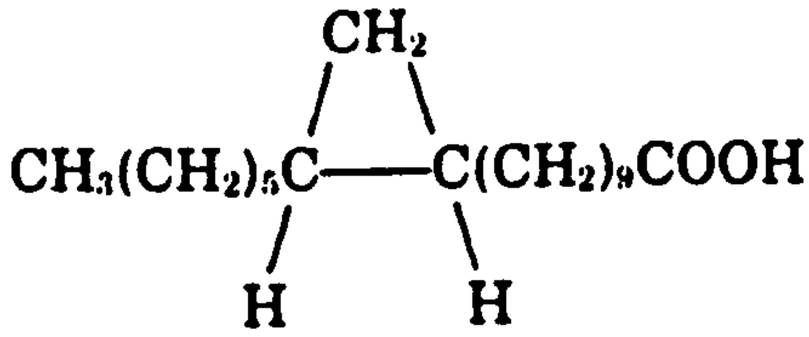
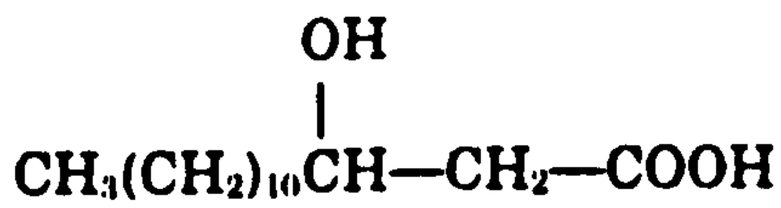
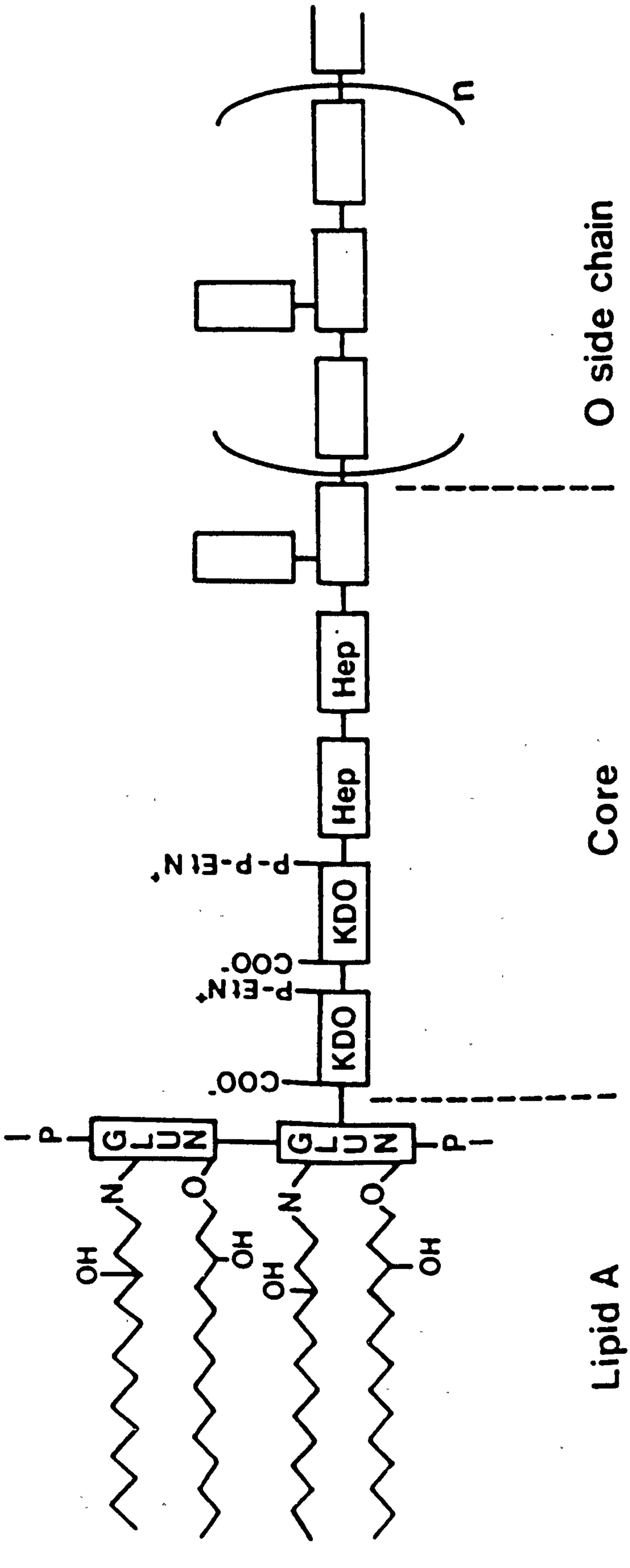
Name (length)	Structure
Major species	
Palmitic (16:0)	$CH_3(CH_2)_{14}COOH$
Palmitoleic (16:1)	
cis-Vaccenic (18:1)	
Minor species	
Lauric (12:0)	$CH_3(CH_2)_{10}COOH$
Myristic (14:0)	$CH_3(CH_2)_{12}COOH$
cis-9,10-Methylene-hexadecanoic (17:0)	
Stearic (18:0)	$CH_3(CH_2)_{16}COOH$
Lactobacillic (19:0)	
Unique to lipopolysaccharide	
3-D-Hydroxymyristic (14:0)	

Fig 1.4 Structures of common fatty acids in bacteria (From Ingraham et al 1983).

Fig 1.5 Generalised structure of bacterial lipopolysaccharide. 'O' side chains and outer core composition vary considerably (From Hammond et al 1984). In E.coli K12 strains the 'O' antigen is lacking (Prehm et al 1976).


O side chain
Core
Lipid A
Hep
Hep
P-P-EtN
KDO
COO
P-EtN
KDO
COO
P
GlcN
GlcN
P
N
O
N
O
OH
OH
OH
OH

a (S)mooth or (R)ough appearance. Analysis of the core structure of the K12 strain of E.coli, used in numerous laboratories and throughout this study, has indicated that it lacks the 'O' antigen and is therefore classified as an 'R' strain (Prehm et al., 1976).

1.2.3 Proteins

The proteins associated with the outer membrane have been intensively studied over the past ten years. Excellent reviews include those by Nikaido & Nakae, (1979); Osborn & Wu, (1980) and Lugtenberg & Van Alphen, (1983). There are twenty to thirty different species of proteins in the outer membrane of Gram-negative cells. Certain of these proteins play a role in the stabilization of the structure and securing the outer membrane to the peptidoglycan layer. In addition, some proteins facilitate the permeation of nutrients and therefore influence the permeability properties of the outer membrane (Rogers et al., 1980 and Nikaido, 1985).

a) Murein lipoprotein

Lipoprotein is involved in stabilizing the outer membrane and anchoring it to the underlying peptidoglycan. It is the most abundant outer membrane protein but is only a small polypeptide of molecular weight 7,200. It is covalently bound to the diaminopimelic acid residue of peptidoglycan, although the majority of copies of the protein exist in the cell envelope in a free (non-attached) form. The isolation of lpp mutants

lacking the gene for both forms of lipoprotein show that it is not essential for the survival of the cell. However, such mutants have severe defects including increased sensitivity to ethylenediaminetetraacetic acid (EDTA) and leakage of periplasmic proteins, which suggests a role for lipoprotein in the stabilization of the outer membrane.

Other outer membrane proteins also have important structural functions. For example OmpA plays an essential role in maintaining cell envelope integrity (see Chapter 5).

b) Diffusion pore proteins (peptidoglycan-associated)

In E.coli 80% of the outer membrane protein content is made up of five different major species (Hall & Silhavy, 1981). Nikaido & Nakae, (1979) have proposed a concept of water filled channels or pores in order to explain how nutrients and solutes of molecular weight 600 or less pass through the outer membrane. The pores (or porins) of Gram-negative bacteria are trimeric aggregates which are in many cases tightly associated with the peptidoglycan layer. Since they also act as receptors for bacteriophages and bacteriocins, the porins must be exposed at the surface as well as opening to the periplasm and therefore completely span the membrane. E.coli K12 contains two major peptidoglycan-associated proteins known as OmpF and OmpC (molecular weight 36.5 and 37K) which have been identified as porins and act as general diffusion pathways for the cell. They are immunologically related to each other (Overbeeke et al., 1980 and Smyth, 1985) and share a similar amino acid composition and N-terminal sequence (Ichihara & Mizushima, 1978). In addition, approximately 10-20 different species of

minor proteins have been identified. Some of these proteins are implicated in iron transport, vitamin B12 uptake or nucleoside transport. However, in most cases the roles of the minor proteins are unknown.

1.3 THE OUTER MEMBRANE AS A PERMEABILITY BARRIER

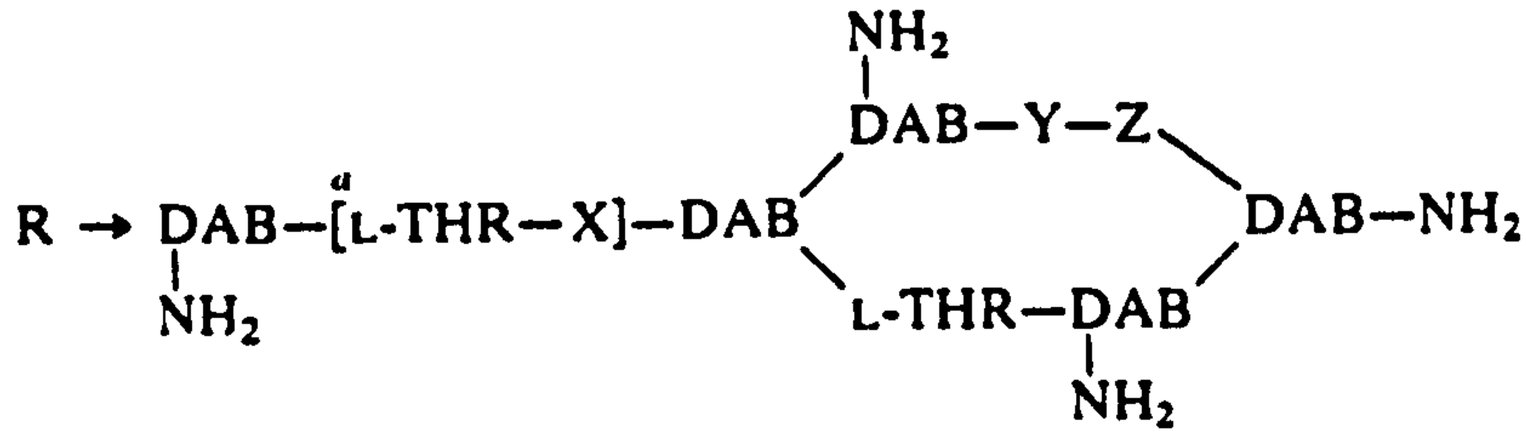
Gram-negative bacteria have an exceptionally efficient barrier against the external environment. The outer membrane by the combination of negative charge and divalent cation cross-bridging of LPS is responsible for the exclusion of a large number of molecules. The presence of the outer membrane ensures that Gram-negative bacteria are resistant to host defence factors such as lysozyme. This enzyme is present in certain body fluids e.g. serum and tears and appears to have unrestricted access to the peptidoglycan of Gram-positive cells, causing them to lyse. The outer membrane of Gram-negative bacteria, however, prevents lysozyme from gaining access to the underlying peptidoglycan and cell lysis fails to occur. More generally, the outer membrane of Gram-negative bacteria is an effective barrier against bile and digestive enzymes for organisms which normally inhabit the mammalian gut (Nikaido & Nakae, 1979). The presence of the barrier is also responsible, in part, for the general lack of susceptibility of Gram-negative bacteria to antibiotics.

The failure of certain antibiotics which are effective against Gram-positive organisms may, however, be ascribed to the inability of the agents to penetrate the Gram-negative

outer membrane (Nikaido, 1976). The cell is able to exclude therefore, many antibiotics such as erythromycin, lincomycin, clindamycin, novobiocin, fusidic acid, nafcillin and cloxacillin (Nikaido & Nakae, 1979). Furthermore, the outer membrane of the cell reduces significantly the penetration of benzyl penicillin, ampicillin, carbenicillin and most of the first and second generation cephalosporins (Nikaido & Nakae, 1979; Richmond & Wotton, 1976; Zimmerman & Rosselet, 1977 and Curtis et al., 1979a & b). Thus, the barrier property of the outer membrane excludes a number of clinically useful antibiotics that might otherwise be used to treat infections caused by Gram-negative organisms. This suggests that should the permeability of the outer membrane be disorganised, then bacteria would become sensitive to an extended range of antibiotics (Vaara & Vaara, 1983a).

1.4 AGENTS ACTIVE AGAINST CELL MEMBRANES

As already noted this thesis has been concerned with the action of membrane-active agents and particularly the polymyxins. The polymyxins (fig 1.6) are produced by Bacillus polymyxa and they exert antibacterial activity predominantly against Gram-negative organisms. The 'natural' antibiotics and semi-synthetic analogues have molecular weights ranging from 1000 to 1200. Characteristically, they are all polycationic decapeptide antibiotics with a fatty acyl 'tail' and five positively charged groups (fig 1.6). The polar head and nonpolar fatty acid chain confer amphipathic properties which



Antibiotic	R	X	Y	Z
Polymyxin A	MeOct	D-DAB	D-LEU	L-THR
Polymyxin B1	MeOct	L-DAB	D-PHE	L-LEU
Polymyxin B2	MeHep	L-DAB	D-PHE	L-LEU
Polymyxin D1	MeOct	D-SER	D-LEU	L-THR
Polymyxin D2	MeHep	D-SER	D-LEU	L-THR
Polymyxin E1 (Colistin A)	MeOct	L-DAB	D-LEU	L-LEU
Polymyxin E2	MeHep	L-DAB	D-LEU	L-LEU
Circulin A	MeOct	L-DAB	D-LEU	L-ILEU
Octopeptin B1	βOHMeDec	—	D-LEU	L-LEU
Octapeptin A2	βOHMeNon	—	D-LEU	L-LEU
Octapeptin A3	βOHDec	—	D-LEU	L-LEU
Octapeptin B1	βOHMeDec	—	D-LEU	L-PHE
Octapeptin B2	βOHMeNon	—	D-LEU	L-PHE
Octapeptin B3	βOHDec	—	D-LEU	L-PHE
Octapeptin C1	βOHMeOct	—	D-PHE	L-LEU

DAB = Diaminobutyric acid
MeHep = 6-Methylheptanoic acid
MeNon = 8-Methylnonanoic acid
MeOct = 6-Methyloctanoic acid
MeDec = 8-Methyldecanoic acid
Dec = *n*-decanoic acid

[a] Octapeptins—omit dipeptide in square brackets.

Fig 1.6 Composition of some polymyxins and octapeptins (From Gale et al 1981).

permit the polymyxins to interact with both LPS and phospholipids (Hsu-Chen & Feingold, 1973).

The ultimate lethal target of polymyxins is generally thought to be the cytoplasmic membrane to which polymyxin binds (Teuber & Bader, 1976). This conclusion was also reached by Newton (1956), when he originally studied the binding of polymyxin B to cells using a fluorescent polymyxin derivative. He found that following fractionation of cells fluorescent activity was located exclusively in the cytoplasmic membrane. In this location the antibiotic rapidly destroys the barrier property of the cytoplasmic membrane, causing leakage of cytoplasmic components and ultimately cell death (Teuber, 1974; Storm et al., 1977). However, for this lethal event to take place polymyxin B must first traverse the outer membrane barrier. Polymyxins are too large to enter the cells through the porin channels (see above) of Gram-negative bacteria (Nikaido & Vaara, 1985). They therefore gain access by disrupting or disorganising the outer membrane (Storm et al., 1977). To do this polymyxin B binds to the outer membrane causing alterations visible by electron microscopy (Schindler & Teuber, 1975; Lounatmaa et al., 1976; and Gilleland & Murray, 1976) and which reflect structural disorganisation of the membrane. Initial binding to the outer membrane appears to involve an interaction of polymyxin B with the LPS (possibly involving the phosphate groups) (Morrison & Jacobs, 1976; Schindler & Osborn, 1979 and Vaara et al., 1979). In addition polymyxin B is known to interact with divalent cation binding sites on LPS (Schindler & Osborn, 1979) causing

displacement of ions by competition rather than chelation. Disturbances in outer membrane stability resulting from such interactions could account for the changes in permeability induced by polymyxin B. The enhancement of permeability can be reversed however by excess magnesium ions, indicating that divalent cation sites are involved in the mode of action of polymyxin B (Cerny & Teuber, 1972 and Newton, 1956).

EDTA is another agent which disorganises the outer membrane of cells and increases permeability. It exhibits strong binding affinity for metal ions such as magnesium. In the presence of Tris(hydroxymethyl)aminomethane (Tris), treatment with EDTA chelates magnesium ions from the outer membrane resulting in the release of specific components such as LPS. This results in a general enhancement of permeability to a number of agents including lysozyme (Leive, 1974). The effects of polymyxin B on the outer membrane differ from EDTA in that the former apparently fails to release LPS into the medium (Vaara & Vaara, 1983b). It has been proposed (Nikaido & Vaara, 1985) that EDTA may act by removing LPS and creating phospholipid bilayer regions in the outer membrane. A significant difference between the two agents is that polymyxin B-treated cells release substantial levels of periplasmic proteins (Cerny & Teuber, 1972) whereas EDTA releases only very small amounts of these proteins.

Other polycationic agents such as protamine and polylysine appear to be more like EDTA with respect to their effects on the outer membrane. They render the outer membrane permeable

to several hydrophobic agents (Katsu et al., 1984a) and bind LPS which is considered to trigger disruption of the permeability barrier (Katsu et al., 1984b). The basic polypeptides (protamine and polylysine) induce substantial LPS release but cause no leakage of periplasmic proteins or visible structural damage to the cell (Vaara & Vaara, 1983b). These workers have also reported that a variety of cations can bind isolated LPS but do not disrupt the barrier function of cells. Therefore, the relationship between peptide structure and permeability increases remains obscure at present.

Unfortunately, both the polymyxins and EDTA are toxic to eukaryotic cells and their usefulness as therapeutic agents is limited (Storm et al., 1977). Successful attempts by Chihara et al., (1973, 1974) to reduce the toxicity of polymyxin E (colistin) towards eukaryotic cells by the removal of the fatty acid tail resulted in the concomitant loss of antibacterial activity in the modified product (colistin nonapeptide). These results prompted a similar approach by Vaara & Vaara, (1983a & b) to produce a non-toxic derivative, polymyxin nonapeptide (PMBN), from polymyxin B. PMBN has little or no antibacterial activity but appears to retain the ability of the parent peptide to disorganise the outer membrane.

Certain aspects of the mode of action of polymyxins are unknown and the results described in this thesis present an extension of our knowledge concerning their action.

CHAPTER 2

MATERIALS AND METHODS

MATERIALS

2.1 CHEMICAL REAGENTS

The majority of chemicals were purchased either from BDH Laboratory Chemicals Ltd., or the Sigma Chemical Co. both located in Poole, Dorset. Polymyxin B Sulphate was purchased from the Calmic Medical Division, Wellcome Foundation Ltd., London and nitrocefin was a gift from Beecham Pharmaceuticals Research Division, Brockham Park, Surrey. ^{3}H labelled L-amino acid mixture (code TRK.440), 6-[^{3}H]-uracil (code TRK.240) and L-[^{35}S]-methionine (code SJ.204) were purchased from Amersham International Plc., Amersham, Buckinghamshire.

2.2 BACTERIA

E.coli K12 3300 pBR322 which produces beta-galactosidase constitutively was used primarily in these studies. The strain was constructed by transformation (Humphreys et al., 1975) of strain 3300 lacI^{-}, lacO^{+}, lacZ^{+}, lacY^{+} (Luria et al., 1960) with plasmid pBR322, which encodes resistance to beta-lactams and tetracycline (Bukhari et al., 1977 and Kopylova-Sviridova et al., 1979). Bacteria lacking this plasmid were used for penicillin binding proteins (PBPs) studies. Cultures were maintained on nutrient agar or Dorset's egg slants, in the dark at room temperature or $4^{0}C$. The E.coli mini cell-producing strain DS410 (Hallewell & Sherratt, 1976) containing pBR322 was also used.

METHODS

2.3 MINIMUM INHIBITORY ANTIBIOTIC CONCENTRATION DETERMINATIONS

Antibiotics were dissolved in sterile distilled water. Minimum inhibitory concentration (MIC) values were determined by serial dilution into nutrient agar (Lab M.Agar, London Analytical Company, Bury, Lancs.). The surface of the dried agar was inoculated with 0.01ml from an undiluted overnight nutrient broth culture (Lab M), using a replicating device to deliver approximately 10^6 colony forming units (cfu). The MIC value was determined after 18h incubation at 37^0C and defined as the lowest concentration of antibiotic to prevent or greatly diminish, visible growth.

2.4 MINIMUM BACTERICIDAL ANTIBIOTIC CONCENTRATION DETERMINATIONS

Antibiotics were serially diluted into nutrient broth which was inoculated with 0.02ml of an overnight broth culture of E.coli to yield an inoculum of approximately 10^6 cfu/ml. A 10ml volume was withdrawn from each tube after 18h incubation at 37^0C and subcultured onto antibiotic-free nutrient agar. The MBC value was determined as the lowest concentration of antibiotic to prevent visible growth after overnight incubation at 37^0C.

2.5 CHECKERBOARD TECHNIQUES TO ASSESS ANTIBACTERIAL COMBINATIONS

Fusidic acid and benzyl penicillin MIC's were determined with liquid cultures using a checkerboard technique (Krogstad & Moellering, 1980) in 'Linbro' multidishes (Flow Laboratories, Irvine, Scotland.). Combinations of antibiotics were tested by the addition of 0.1ml of appropriately diluted fusidic acid or benzyl penicillin and 0.1ml of diluted PMBN. Each well was inoculated with exponentially growing cells of E.coli in 1.8ml of nutrient broth (10^6cfu/ml). Thus, individual wells contained a unique combination of each antibiotic tested and PMBN. Multidishes were incubated at 37^0C overnight and growth recorded as turbidity.

2.6 DETERMINATION OF VIABLE NUMBERS OF BACTERIA FOLLOWING EXPOSURE TO POLYMYXINS

E.coli K12 3300 (pBR322) cells were grown in nutrient broth (LabM) to mid-exponential phase and the cells harvested by centrifugation at 8K/10 min/4^0C. The cells were washed twice in 10mM sodium phosphate buffer pH 7.4 and resuspended in the same buffer to give a range of cell densities from 10^{10} to 10^6cfu/ml. Aliquots of each suspension were treated at time zero with either polymyxin B (200ug/ml), PMBN (200ug/ml) or remained untreated and were gently agitated at 37^0C in a waterbath. Samples were removed at timed intervals, rapidly diluted in nutrient broth and plated onto nutrient agar to determine viable bacteria according to the method of Miles et al. (1938). It was necessary to dilute cultures containing high concentrations of polymyxin B by at least $1/10^4$ to obviate the "carry-over" effects of the antibiotic.

2.7 PREPARATION OF PMBN AND ITS CHARACTERIZATION

The proteolytic enzyme ficin was used to prepare PMBN from polymyxin B as described by Chihara et al., (1973, 1974). Polymyxin B (2g) was dissolved in 10mM sodium phosphate buffer (pH 7) and mixed with an equal volume of the same buffer containing 1.5g of ficin (Sigma Chemical Co.). The mixture was incubated overnight at 37^0C with shaking. Ficin residue was destroyed by boiling for 5 min and removed by centrifugation (3K/10 min). The resulting supernatant was subjected to solvent extractions in both acidic and alkaline conditions. The supernatant was adjusted to pH 2 with concentrated hydrochloric acid, mixed in a separating funnel and 0.5 x volume n-butanol added. Following three extractions, the aqueous layer was adjusted to pH 8 with NaOH and alkali extraction performed three times with n-butanol. Finally the aqueous phase was neutralised by the addition of 1M HCl and layered onto an ion exchange column consisting of Amberlite resin IRA 410 (BDH). The aqueous phase was collected, frozen and freeze-dried for 8-12h. PMBN prepared by this method was characterised by thin layer chromatography (TLC), high-pressure liquid chromotography (HPLC), nuclear magnetic resonance (NMR) and spectral analysis. TLC was carried out as previously described (Vaara & Vaara, 1983a). Prepared TLC sheets (Eastman Chromatogram cellulose sheets 13255, Kodak) were spotted and run for 4h in butanol/acetic acid/water (60/20/20). The plates were developed with ninhydrin solution and the results recorded. A small sample of the freeze-dried material was used

for HPLC analysis. HPLC was performed on a Waters u Bondapak C18 (P/N 84042 S/N) column [30cm x 3.9mm (internal diameter)] eluted with methanol - 0.1M phosphate buffer (pH 3) (1.5 : 8.5, v/v) at 3.0ml/min. The chromatographic equipment consisted of a Waters model 6000A pump, Rheodyne injector fitted with a 20ul loop and a variable-wavelength UV detector set at 210 nm for detection of polymyxin and PMBN (Bird et al., 1984). The retention time for 20ul of each solution was measured. . Spectral analysis of the compounds was performed with a Pye Unicam PU8800 spectrophotometer linked to an Acorn microcomputer loaded with Pye Unicam scanning software. Each compound was prepared at 10mg/ml of distilled water and tested in 1cm cuvettes.

2.8 ENZYME,PROTEIN, AND 3-KETODEOXYOCTONATE ASSAYS

2.8.1 Beta-lactamase

Beta-lactamase (EC 3.5.2.6) was assayed using the chromogenic cephalosporin nitrocefin (O'Callaghan et al., 1972). Samples (50ul) containing amounts of beta lactamase were diluted in 0.95ml of 10mM sodium phosphate buffer (pH 7) and placed in 3ml cuvettes maintained at 37^0C in a cell preheater (Pye-Unicam Ltd.,Cambridge). Cuvettes were transferred to a temperature controlled cell holder in a PU8800 spectro-photometer (Pye-Unicam Ltd.) and 2ml of nitrocefin (50ug/ml) added. The change in absorbance at 482nm/min was recorded and the amount of substrate destroyed was calculated by applying the formula below:

$$\text{umoles nitrocefin destroyed /min/ml of enzyme} = \frac{\text{Abs}_{482}\ /\text{min.}}{1.59} \times V \times 0.3$$

where "V" = conversion to 1ml

2.8.2 Beta-galactosidase

Beta-galactosidase (EC 3.2.1.23.) was assayed by its ability to liberate o-nitrophenol (ONP) from o-nitrophenyl-B-D-galactopyranoside (ONPG) (Chopra et al., 1977). To assay for beta-galactosidase in the experimental samples, 50ul was taken, diluted with 0.95ml of 10mM sodium phosphate buffer (pH 7.4) and pre-incubated for a few minutes at 37^0C. A freshly prepared solution of ONPG (4ml) in 10mM sodium phosphate buffer (pH 7.4) was added to the sample and incubated at 37^0C until the yellow colour of ONP developed (approximately 15-30 min). The reaction was stopped by the addition of 5ml of a 0.5M solution of sodium carbonate. The absorbance of the solution was measured against a reagent blank at 420nm in a spectrophotometer. The amount of ONP liberated from ONPG was assessed by reference to a standard curve of ONP concentrations (range 0.1-2 umoles) prepared in appropriate volumes of 10mM sodium phosphate buffer (pH 7.4) against absorbance at 420nm.

2.8.3 3-Ketodeoxyoctonate

3-ketodeoxyoctonate (KDO) was measured by the method of Karkhanis et al., (1978). 50ul aliquots containing unknown amounts of KDO were diluted in 0.45ml of 10mM sodium phosphate (pH 7.4) and placed in Eppendorf centrifuge tubes. To each

tube 0.5ml of 0.4N sulphuric acid was added and the samples heated at 100^0C in a waterbath for 30 min to release KDO. The tubes were spun in a microfuge (3 min) and 0.5ml of the supernatant removed into bijoux bottles. The mixture was then treated with 0.25ml of a solution of 0.04M periodic acid in 0.125N sulphuric acid, left at room temperature for 20 min and then 0.25ml of a 2.6% solution of sodium arsenite in 0.5N hydrochloric acid was added. Finally, the addition of 0.5ml of a 0.6% solution of thiobarbitic acid produced a red chromophore. The tubes were heated at 100^0C for 15 min and the chromophore extracted, whilst hot, with 1ml dimethyl-sulphoxide (DMSO). The absorbance of the samples was read at 548nm against the reagent blank. A set of standard KDO solutions ranging in concentration from 10-50ug KDO per tube were prepared and subjected to the above procedure. The amount of KDO in the material was calculated from the standard curve.

Standard lines were drawn for beta-galactosidase and keto-deoxyoctonate calibrations using a computer program (Chopra, 1985b) for regression analysis. The coefficient of correlation was calculated for each line and those lines displaying poor correlation rejected.

2.8.4 Proteins

Protein was assayed according to the method of Bradman (1976) using a 'Protein Assay Kit' (Bio-Rad Laboratories Ltd., Watford). The assay is based on the colour change of a dye in response to various concentrations of proteins. Initially a protein standard curve was constructed by dilution of bovine

serum albumin in 10mM sodium phosphate buffer (pH 7.4) to give a range of concentrations from 10-140ug per tube. Dye reagent concentrate was diluted and filtered according to the manufacturers instructions and 5ml added to each tube containing 100ul of each of the standard protein concentrations. After 5 min, the absorbance of the solutions was read at 595nm with a spectrophotometer. (The colour is stable between 5-60 min). Plots of absorbance (595nm) versus protein concentration (ug) were drawn using a computer program for regression analysis (Chopra, 1985b). The coefficient of correlation was calculated as before and those lines displaying poor correlation rejected. Experimental samples (100ul) were treated as above; 5ml of dye reagent added and absorbance readings at 595nm recorded. The amounts of protein in each sample were estimated from standard curves with correlation coefficients >0.99.

2.9 PREPARATION OF BACTERIAL CELL FRACTIONS.

2.9.1 Separation of inner and outer membranes.

Inner (cytoplasmic) and outer membranes of E.coli K12 3300 (pBR322) were separated either by Sarkosyl treatment to solubilise the inner membrane (Filip et al., 1973 and Chopra & Shales, 1980) or by using a sucrose density gradient method (Yamato et al., 1975). To prepare envelopes for Sarkosyl treatment, cells in the early logarithmic phase of growth were harvested at 6K/5 min/4^{0}C washed in 10mM sodium phosphate buffer (pH 7.4) and then resuspended in 15ml of this buffer.

Following disruption by sonication (4 x 20 sec with 30 sec cooling periods), unbroken cells were removed by low speed centrifugation (3K/30 min/4^{0}C) and crude envelopes isolated from the supernatant by high speed centrifugation (40K/60 min/4^{0}C). Cell envelopes were resuspended in 0.5%(v/v) Sarkosyl (N-lauroyl sarkosine-sodium salt) (Filip et al., 1973). Sarkosyl insoluble fractions containing outer membrane proteins were isolated by centrifugation (40K/2h/4^{0}C), washed with buffer and stored at -20^{0}C. Proteins in the Sarkosyl-soluble fraction were precipitated with cold acetone, collected by centrifugation, dried at room temperature overnight and stored at -20^{0}C. Separation of inner and outer membranes on sucrose gradients (Yamato et al., 1975) was achieved from cells in which periplasmic and cytoplasmic fractions had been removed (see below). Crude envelopes were suspended in 40ml of 3mM EDTA (pH 7.2) and centrifuged at 40K/60 min/4^{0}C. The envelopes were washed, resuspended in 8ml of 3mM EDTA (pH 7.2) and layered over a cushion of 3mM EDTA containing 52% (w/v) sucrose. The envelopes were centrifuged at 40K/12-18h/4^{0}C and the upper band containing inner membrane was removed. To remove contaminating whole cells from the outer membrane preparation, the pellet was resuspended in 3mM EDTA containing 10% (w/v) sucrose and layered on to a sucrose step gradient constructed from 64% (w/v) sucrose (6ml) and 9ml of 70% (w/v) in 3mM EDTA. The gradients were spun to equilibrium (40K/24-48h/4^{0}C). The material containing outer membrane proteins was located at the interface of the 64-70% layers and contaminating whole

cells were pelleted.

2.10 ISOLATION OF PERIPLASMIC AND CYTOPLASMIC FRACTIONS

The procedure of Yamato et al., (1975) was followed to isolate proteins from the periplasm and cytoplasm. Bacteria were grown to the early logarithmic stage of growth in nutrient broth and harvested. Cells were cooled to 4^0C, washed and resuspended in 16ml of 30mM Tris-HCl (pH 8) containing 20% (w/v) sucrose (TS 20) to give approximately 10^{11} bacteria per ml. The cells were treated with solutions of 7mg/ml lysozyme in TS20 (0.176ml) and 0.1M EDTA in TS20 (1.6ml). The solutions were added rapidly and simultaneously to cells maintained on ice, gently mixed and left on ice for a further 20-30 min. The spheroplasts were collected by centrifugation at 8K/30 min/4^0C and the supernatant containing periplasmic proteins and lysozyme was subjected to dialysis overnight at 4^0C against deionised water and freeze-dried. Spheroplasts were resuspended in 16ml of 3mM EDTA pH 7.2 containing 20% (w/v) sucrose (ES20) and sonicated for 6 x 20 sec bursts with 30 sec cooling intervals, diluted in 3mM EDTA (pH 7.2) and spun at 40K/1.5h/4^0C. The supernatant containing the cytoplasmic fraction of the cell was removed, dialysed overnight against deionised water at 4^0C and freeze-dried.

2.11 OTHER METHODS FOR THE SELECTIVE RELEASE OF PERIPLASMIC PROTEINS

Methods for the release of periplasmic proteins from E.coli other than treatment with EDTA/lysozyme (see above) include osmotic shock techniques. In general, bacteria are first suspended in a concentrated solution of sucrose in EDTA and then removed to a medium of low osmotic strength. Two methods were used. The osmotic shock procedure of Neu & Heppel (1965) and Heppel (1968) were followed: Bacteria were grown in nutrient broth as before, harvested by centrifugation at 8K/20 min/4^0C. The cells were washed twice in a cold solution of 0.01M Tris-HCl (pH 7.1) and 0.03M NaCl. The resulting cell pellet was resuspended in a solution of 0.03M Tris-HCl (pH 8.0) and 10^{-4}M EDTA containing 20% sucrose (w/v) maintained at room temperature. The cells were collected by centifugation (8K/10 min) and then rapidly dispersed in cold deionised water. The mixture was agitated for 10 min at 4^0C. Release of periplasmic proteins occurred in the cold water wash. The mixture was centrifuged at 8K/20 min/4^0C the supernatant ('shock fluid') removed and freeze-dried. An alternative method for obtaining periplasmic proteins is by the modified osmotic shock procedure of Willis et al., (1974). Cells were harvested at 15^0C and washed in a solution containing 0.03M Tris-HCl (pH 7) and 0.03M NaCl. Cells were resuspended in 0.03M Tris-HCl (pH 7) and the cells maintained at room temperature. An equal volume of 0.03M Tris-HCl - 2mM EDTA containing 40% sucrose was added. The cells were collected by centrifugation (8K/20 min/15^0C) and the plasmolysed cell pellet resuspended in 20ml of cold dilute $MgCl_2$ solution (10mM). The mixture was centrifuged at 8K/20 min/4^0C, the

supernatant ('shock fluid') removed and after the addition of 0.02ml of 2-mercaptoethanol the material was freeze-dried.

2.12 ELECTROPHORESIS AND DETECTION OF POLYPEPTIDES

2.12.1 One dimensional polyacrylamide gel electrophoresis

a) Sample preparation

One dimensional electrophoresis of both labelled and non-labelled proteins was performed in slabs of polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS). All samples were prepared for electrophoresis by solubilization in the sample preparation buffer (SPB), similar to that described by Laemmli (1970), containing 0.2M Tris-HCl (pH 6.8), 9.1% glycerol, 4.5% 2-mercaptoethanol, 1.3% SDS and 0.001% bromo-phenol blue. The temperature and time of solubilization routinely used were 100^0C for 5 min unless otherwise indicated. Freeze-dried samples or cell fraction pellets were resuspended in SPB, heated for 5 min at 100^0C, centrifuged and 50 - 100ul of the protein containing supernatant added to the gel.

b) Fixed concentration running gels

Gels containing 10% acrylamide were prepared from a stock solution of 29% by weight of acrylamide and 1% by weight of N,N'-bis-methylene acrylamide. Separating gels were composed of the following:- 16.5ml of 0.75M Tris-HCl (pH 8.8), 11ml of 30% acrylamide-bis-acrylamide solution, 0.5ml of 10% freshly prepared ammonium persulphate, 0.35ml of 10% SDS and 4.6ml of

deionised water per gel. The gels (33ml volume) were polymerised chemically by the addition of 10ul of tetra-methylethylenediamine (TEMED).

c) Gradient running gels

Alternatively, linear gradient gels (5-20% acrylamide slabs) were prepared by following exactly the method described by Hames (1981).

d) Stacking gels

The stacking gel (2cm long) for both 10% and gradient gels consisted of 5% acrylamide containing 4.2ml of 0.25M Tris-HCl (pH 6.8), 130ul of 10% ammonium persulphate, 83ul of 10% SDS, 2.5ml of deionised water and each gel polymerised by the addition of 3ul TEMED.

e) Electrophoresis

Electrophoresis was performed on slab gels (18 x 16 x 0.15cm) in a vertical electrophoresis tank (Bio-Rad Ltd.) in cooled (15^0C) electrode buffer (pH 8.3) containing 0.025M Tris-HCl, 0.192M glycine and 0.1% SDS (Laemmli, 1970). Polypeptides were separated by the application of 30mV per gel with maximum voltage until the blue marker had run the complete length of the gel.

f) Detection of proteins and estimation of polypeptide molecular weight

Non-radioactive proteins were detected by staining the gel with

Coomassie brilliant blue R250 (Hames, 1981) and then destaining it by repeated washing in a solution of 10% glacial acetic acid and 10% isopropanol. Labelled proteins were detected by auto-radiography under conditions where image intensity is directly related to the radioactivity in separated protein bands. The following proteins were used as molecular weight standards: Lysozyme (14K), carbonic anhydrase (29K), alcohol dehydrogenase (37K), fumarase (49K), bovine albumin (68K) and beta-galactosidase (130K) (all from Sigma). A mixture was prepared containing approximately 2mg of each of the above proteins in 1ml of the sample buffer prepared as above, 10-20ul applied to the gels and used to estimate the molecular weights of uncharacterised proteins. Scanning densitometry of separated polypeptides was performed with a Joyce Loebl Chromoscan 3 equipped with automated peak integration facilities.

2.12.2 Two dimensional polyacrylamide gel electrophoresis

Two dimensional polyacrylamide gel electrophoresis (PAGE) was performed according to the method of Ames & Nikaido (1976) based on the procedure of O'Farrell (1975).

a) Solubilization

The solubilization procedure of Ames & Nikaido (1976) was followed. Protein (3mg) was solubilized at 100^0C for 5 min in 200ul of SPB containing 0.1ml of 0.25M Tris HCl (pH 6.8), 0.2ml of 10% SDS, 0.5ml of 1mM magnesium acetate, 0.1ml water and 0.1ml 2-mercaptoethanol. Eppendorf centrifuge tubes

containing samples were spun for 3 min (Microfuge, MSE, Crawley, Sussex) and 300ul of sample dilution buffer (SDB) containing 9.5M urea, 0.5ml Ampholine (pH 3.5-10) (LKB Ltd. London), 0.8ml Triton X100, 0.5ml 2- mercaptoethanol in 7.7ml of water added (similar to Ames & Nikaido (1976) but NP-40 replaced by Triton X100). Solid sucrose (40%) was added to the final mixture and the samples kept cool until they were loaded onto gels.

b) Isoelectricfocusing

The first dimension (isoelectricfocusing) is as described by O'Farrell (1975). Gels composed of 2.75g urea, 0.7ml of 30% acrylamide solution, 1ml of Triton X100, 1ml of water, 0.25ml of Ampholine (pH 3.5-10) and 5ul of 10% ammonium persulphate were prepared in narrow bore glass tubes, 5ul of TEMED were added and the gels overlayed with 8M urea before polymerisation occurred. The tubes were mounted in a cylindrical gel apparatus and after removal of the urea, the samples were placed into the tubes by pipetting 50-150ul with a Hamilton syringe. Ten to twenty ul of the sample overlay solution (O'Farrell, 1975) were added to the tubes which were then filled with cathode electrode solution 0.02M sodium hydroxide (degassed by boiling). The anode reservoir was filled with 0.01M H_3PO_4. Electrophoresis was performed at 400V for 18-20h. The cylindrical gels were removed from the glass tubes, equilibrated in a solution containing 125ml of 0.25M Tris HCl (pH 6.8), 50ml of glycerol, 25ml of 2-mercaptoethanol, 10g SDS and water to 500ml and then stored frozen. A linear

gradient gel (5-20% acrylamide) was prepared as before except that the stacking gel was 2.5cm high and its top surface was flat. Equilibrated cylindrical gels were applied to the second dimension gel by placing them on the top buffer tank of the slab gel apparatus (Bio-Rad Ltd.). Hot (50-60^{0}C) agarose (1% in equilibration buffer) was pipetted below and on top of the cylindrical gel taking care not to entrap air bubbles and electrophoresis was then performed at 30mA for 4-5h. Gels were either stained with Coomassie blue as before, or if the separation involved labelled protein, the dried gel was autoradiographed or fluorographed.

2.12.3 Immunoblotting

Samples containing outer membrane proteins were separated on 10% acrylamide gels as before and transferred on to nitrocellulose paper by the method of Towbin et al (1979). Following electrophoresis the gel was removed from the glass plates, equilibrated in buffer containing 0.025M Tris-HCl, 0.192M glycine and 20% methanol for 30 min and proteins were transferred by positioning the gel on to a pre-wetted sheet of nitrocellulose paper (Bio-Rad Ltd.) in a "transblot" apparatus (Bio-Rad Ltd.) according to the manufacturer's instructions. Transfer was performed at 30V overnight and then 60V for one hour. The nitrocellulose paper was removed from the apparatus and to check if adequate transfer of protein had occurred the paper was soaked in a solution of 0.1% amido black, 45% methanol and 10% glacial acetic acid. The paper was destained with a solution of 90% methanol and 2% glacial acetic acid.

To examine which proteins had been transferred, the "donor gel" was stained with Coomassie blue solution as previously described. Characterisation of the transferred proteins was carried out by a modification (Anwar et al., 1984) of the procedure described by Towbin et al., (1979). After electrophoretic transfer of the proteins, the nitrocellulose paper (not stained with amido black) was incubated with TBS (10mM Tris-HCl in normal saline pH 7.4) containing 0.3% Tween 20 for one hour to saturate the non-specific binding sites. The paper was saturated in antisera (anti-OmpF/C) diluted 1 in 8 in TBS for 4 hours at 37^0C and then further incubated for 2 hours with horseradish peroxidase goat anti-rabbit IgG conjugate (Sigma) diluted 1 in 2000 in TBS. Following incubation, the paper was washed thoroughly with TBS and visualised by using a solution of 25ug/ml of 4-chloro-1-naphthol in 10mM Tris-HCl containing 0.01% hydrogen peroxide.

2.13 PENICILLIN BINDING PROTEINS

2.13.1 Labelling of penicillin binding proteins (PBPs) in cell envelopes treated with PMBN

Two litres of bacteria were grown in nutrient broth to early logarithmic phase, cooled and harvested by centrifugation (9K/10 min/4^0C). Bacteria were resuspended in 60ml of 50mM sodium phosphate buffer (pH 7) and 0.75ml of dithio-threitol solution (7.7 mg/ml) was added to each 15ml of resuspended cells. Bacterial suspensions (15ml) were sonicated (4 x 20sec) with cooling periods of 30 sec and unlysed bacteria

removed by slow centrifugation (3K/30 min/4^{0}C). The pellet containing envelopes was washed several times in cold 50mM sodium phosphate buffer (pH 7). The protein concentration of the envelopes was adjusted to approximately 10mg/ml and the envelopes dispensed in 1ml aliquots in Eppendorf centrifuge tubes. The envelopes were incubated with PMBN (200ug/ml final concentration) for 60 min at 37^{0}C with gentle shaking. Controls without PMBN treatment were included. The envelopes were recovered by high speed centrifugation (40K/60min /4^{0}C) and resuspended in buffer. Aliquots (100ul) were treated for 15 min at 37^{0}C with benzyl [^{14}C] penicillin (50 or 100uM concentration) as described by Spratt (1977) and the reaction was terminated by the addition of 10ul of non-labelled benzyl penicillin (120mg/ml) and 5ul of 20% Sarkosyl. Insoluble material was removed by centrifugation (Microfuge, 20 min). Equal volumes of supernatant and x 2 concentrated SPB containing 300mg SDS, 7ml of 0.25M Tris-HCl (pH 6.8), 3ml glycerol and 2ml of 2-mercaptoethanol were mixed. Finally, the samples were heated in the SPB for 5 min at 100^{0}C, spun (Microfuge, 1 min) and the supernatant (50 or 100ul) loaded onto gels.

2.13.2 Gel electrophoresis for separation of PBPs

PBPs were separated by slab gel electrophoresis according to the procedure of Suzuki et al (1978). Acrylamide gels (18 x 16 x 0.15cm) were prepared from a stock solution of 29.5% (w/v) acrylamide and 0.5% (w/v) bis-acrylamide. The separating gel consisted of 16.5ml of 0.75M Tris-HCl (pH 7.8), 8.25ml of stock

acrylamide solution, 7.35ml of deionised water, 0.5ml of fresh ammonium persulphate and 0.35ml of 10% SDS. The gel was polymerised with 10ul of TEMED. The stacking gel was prepared as before. Electrophoresis was at 30mV for 4 - 5 hours. After electrophoresis the gels were fixed in 10% acetic acid for a 1-2h, washed thoroughly in water and prepared for fluorography by soaking for 30 min in a solution of 1M sodium salicylate (Chamberlain, 1979). Gels were dried and placed in cassettes containing preflashed X-ray film. Cassettes were left for 6 weeks at -50^{0}C before development of films. Labelled PBPs were quantified by densitometry using the Joyce Loebl Chromoscan 3.

2.14 LEAKAGE OF SMALL CYTOPLASMIC CONSTITUENTS FROM E.COLI

The loss of various low molecular weight cytoplasmic constituents from E.coli after exposure to polymyxin B and PMBN was investigated.

2.14.1 Leakage of amino acids

Loss of free amino acids from the cytoplasmic pool was investigated with ^{3}H-labelled mixed amino acids using methods first introduced by Britten & McClure (1962). Bacteria were grown to early logarithmic phase and then incubated for 60 min with chloramphenicol (100mg/ml) and ^{3}H amino acids (2uCi/ml) to form a labelled amino acid pool. Bacteria were harvested, washed and resuspended in 10mM sodium phosphate

buffer (pH 7.4) to give a cell density of approximately 10^{10}/ml. The cell suspension was divided into equal aliquots and treated with the appropriate concentrations of polymyxin B or PMBN. Samples were removed at regular time intervals and processed rapidly to remove bacteria (Microfuge, 3 min). To obtain a "total count", suitable volumes (250ul) of the supernatant were mixed with 20ml of a scintillant consisting of 2-(4-tert-butylphenyl)-5-(4-biphenylyl) 1,3,4 -oxadiazole (butyl-PBD 6g), Triton X100 (500ml), toluene (1l) and deionised water (150ml). The total radioactive count represents both free labelled amino acid and radioactivity incorporated into protein. An equal volume of the supernatant was dispensed into cold 10% trichloroacetic acid solution (TCA), maintained on ice for 30 min and filtered through GF/C glass fibre filters (Whatman 2.5cm). Filters were washed with two volumes of 10% TCA and three volumes of 1% acetic acid, dried and placed in scintillation vials. 10ml of a scintillant consisting of butyl-PBD without Triton were added. The radioactivity of the TCA precipitate is a measure of incorporation of labelled amino acids into protein. Thus, the radioactivity released from the soluble pool was calculated by subtracting the quantity of labelled amino acids incorporated into protein from the total (Britten & McClure, 1962).

2.14.2 Leakage of uracil

Release of free (pool) uracil from E.coli was investigated with 6-^{3}H labelled uracil. Bacteria were grown to early logarithmic phase and then incubated with 6-^{3}H-uracil

(2uCi/ml) for 45 min to form a labelled uracil pool. The bacteria were harvested and exposed to antibiotics, samples removed at regular intervals and processed as described in section 2.14.1. Free labelled uracil in the cell-free supernatants was quantified according to the method of Dougherty & Saukkonen (1985), which is essentially similar to the method used above to quantify loss of pool amino acids.

2.14.3 Release of potassium ions

Potassium leakage was measured essentially according to Lannigan & Bryan (1985) using a model 93-3199 potassium specific electrode (Russell Ltd. Auchtermuchty, Scotland.) coupled to a Digital 110 millivolt recording meter (Corning Scientific Instruments, Medfield, Massachusetts). The potassium electrode was calibrated and used according to the manufacturer's instructions; a calibration curve was constructed in the presence of 0.01M NaCl to give a suitable background ionic strength for low level measurements. A solution of potassium chloride (0.1M) in deionised water was prepared and diluted to give a range of concentrations from $1x10^{-3}$M to $1x10^{-6}$M. Appropriate volumes of "ionic strength adjuster" (NaCl) were added and the electrode potential (in mV) recorded for each concentration. It was confirmed that plots of potassium chloride concentration (log) against electrode potential were linear (data not shown). Bacteria were grown in nutrient broth, harvested at room temperature and washed in 10mM sodium phosphate buffer (pH 7.4). The cells were resuspended in the buffer to give

approximately 10^{10} cells/ml. Aliquots (50ml) of the cell suspension were taken and equilibrated at 37^{0}C for 10 min. Suitable volumes of the antibiotic under test were added rapidly to the cells and mV readings taken at frequent intervals until no further change was noted. The concentration of potassium released was determined from the low-level calibration curve.

2.15 ELECTRON MICROSCOPY

2.15.1 Preparation of cells for transmission electron microscopy

a) Treatment of cells with antibiotics

E.coli K12 3300 (pBR322) were grown to mid-logarithmic phase in nutrient broth, harvested by centrifugation at 3K/10 min/ 4^{0}C, washed twice in 100mM filtered cold sodium phosphate buffer (pH 7.4) and then resuspended in the same buffer to a cell density of 10^{10}/ml. The cell suspension was divided into three aliquots. At time zero polymyxin B (200ug/ml) was added to one portion and PMBN (200ug/ml) to the second. The third aliquot served as an untreated control. Bacteria were incubated at 37^{0}C with shaking on an orbital shaker (50-75rpm). Samples were removed at 0, 15, 30 and 60 min after the addition of antibiotic, centrifuged at 3K/5 min and washed in 100mM sodium cacodylate buffer (pH 7.2).

b) Fixation

Cells were resuspended in the same buffer containing 2.5% glutaraldehyde (E.M.grade) and left to fix at room temperature

for at least one hour. The cells were spun at 3K/5 min at room temperature, washed twice in 100mM sodium cacodylate buffer and treated with 1% osmium tetroxide (TAAB Laboratory Equipment Ltd., Reading) for 1 hour. The cells were spun at 3K/ 5 min and stored at 4^0C.

c) Processing of cells using bovine serum albumin (BSA)

Osmium-treated cells were resuspended in a solution of 50mM Tris-HCl/0.15M NaCl (pH 7.2), spun at 3K/5 min and resuspended in 0.2ml of a solution of 50mM Tris-HCl with 4% BSA (pH 7.2). To form a solid pellet of cells, 20ul of 25% glutaraldehyde was added, the suspension mixed and quickly transferred to a Beem capsule (size 00 G363-1 hyperbolic, Agar Aids Ltd., Cambridge Road, Stanstead, Essex). The Beem capsule was placed within a centrifuge tube and spun at 4K for at least 5 min. The pellet at this stage had formed a cross-linked gel, which encapsulated the bacterial cells and could be removed from the capsule and sliced thinly into 1mm curved pieces in 70% alcohol. The samples were dehydrated with 90% alcohol and transferred through three changes of 100% alcohol (silica-gel dried). The samples were transferred initially to 100% propylene oxide (10 min), then to a solution containing 50% propylene oxide and embedding resin (TAAB Laboratories Ltd.), for 30 min and thirdly to a solution containing 25% propylene oxide and resin (30 min). Finally samples were transferred to 100% resin and left overnight with gentle agitation on a rotator (Type N, TAAB Laboratories Ltd.). The samples were transferred to fresh resin for 4 - 5 hours and then transferred to resin filled Beem capsules (size 00 G360-1 TAAB Laboratories Ltd.) and

polymerised overnight at 60^0C. Embedding resin was prepared by mixing 50g of resin with hardeners; 38g of Dodecenyl succinic anhydride (DDSA) and 12g of Methyl nadic anhydride (MNA). The polymerisation catalyst DMP-30 was added to the mixture (1.8g).

d) Thin section cutting

Knives for cutting ultra thin sections were prepared from glass strips (25 x 5mm., LKB Bromma 7890-04) with a knifemaker (LKB Type 7801B). Thin sections (1 micron thick) were cut from trimmed resin with the LKB Ultrotome III and collected in a 'truf' (LKB 2280-100) containing filtered water. The sections were heat fixed on a glass slide and stained with warmed 0.25% Azure II and 0.5% borax solution, dried and examined under the light microscope. For ultra thin sections, the automatic feed system on the Ultrotome was employed. This allowed the machine to advance 70nm at a time and the thickness of sections could be assessed by optical interference. Sections demonstrating silver or gold were of an optimal thickness, whereas purple sections were not acceptable. Ultra thin sections were mounted on copper/rhodium grids for electron microscopy (HR25;300 mesh x 3mm, Graticules Ltd., Tonbridge, Kent). The sections were allowed to dry on the grids to await staining.

e) Staining for the Transmission Electron Microscope (TEM)

A saturated solution of uranyl acetate in 70% alcohol was shaken well and filtered through a 0.2u disposable filter (Flow Laboratories Ltd., Irvine, Scotland). A few drops were placed on parafilm and the grid (surface containing sections in liquid) left in the dark for 15 - 20 min at room temperature.

Subsequently, the grid was washed, first with 70% alcohol and then with double deionised water. Reynolds lead citrate was prepared as shown below, filtered and a few drops placed on parafilm in a petri dish containing filter paper wetted with sodium hydroxide (NaOH) to absorb the carbon dioxide. The grid containing sections were placed on the lead citrate solution for a maximum of 10 min, washed with 0.02M NaOH and then with double deionised water. The grids were blotted and left to dry. The sections were examined with a Philips 300 Transmission Electron Microscope and micrographs taken with a 35mm camera using Kodak fine grain positive film unperforated (Agar Aids Ltd.). Reynolds lead citrate was prepared by adding 30ml of freshly boiled deionised water to 1.33g of lead nitrate and 1.76g of sodium citrate, mixed and placed at room temperature for 30 min. A 1M solution of NaOH (8ml) was added and the solution made up to 50ml with boiled water.

2.15.2 Scanning electron microscopy

a) Treatment of cells with antibiotics

For scanning electron microscope (SEM) studies, bacteria were either grown, harvested and resuspended to give a cell density of 10^{10} cells/ml and treated with antibiotics as described in 2.15.1, or alternatively 20ul of an undiluted overnight broth culture were added to 2ml of filtered nutrient broth containing the appropriate concentration of antibiotic. Bacteria were incubated at 37^{0}C without shaking and samples removed at 0, 15 and 60 min. Cells were fixed in 2.5% glutaraldehyde and 1% osmium tetroxide as described previously

for TEM fixation. After fixing, cells were washed in 100mM sodium cacodylate buffer (pH 7.2), resuspended and stored in this buffer.

b) Preparation for SEM

Apparatus containing a 0.4um cellulose acetate filter (13mm diameter, Sterilin Ltd., Feltham, Middlesex) was assembled and the cells collected on the filter. The filter was dehydrated through a series of alcohol washes in a 24 well plastic multidish (Flow Laboratories Ltd.). The filter was carefully transferred through 30%, 50%, 70%, 80%, 90% and two transfers in 100% ethanol, allowing 5 min in each solution. Using a small filter paper folded into the shape of an envelope, which had been soaked in ethanol, the filter was transferred, under ethanol, and secured with a staple. This procedure allowed the filter containing the cells to be kept flat and allowed exchange of fluids to take place. The envelopes at this stage were subjected to critical point drying after which, specimens were retrieved and mounted, using double sided sellotape, onto aluminium stubs (Polaron Equipment Ltd., Watford, Herts.). The filters were coated with platinum using a sputter coater (Emscope Ltd., Ashford, Kent) and examined with a Philips 501 Scanning Electron Microscope. Photographs were taken with Ilford FP4 film.

CHAPTER 3

CHARACTERISATION OF POLYMYXIN B NONAPEPTIDE

INTRODUCTION

The work reported in this thesis has been directed primarily to obtaining a better understanding of the mode of action of PMBN (see subsequent chapters). The purpose of the experiments described in this chapter have been to establish that PMBN of the highest purity could be prepared. Clearly this is an essential prerequisite before conducting detailed studies on its mode of action.

Reasonable quantities of polymyxin B nonapeptide were produced from commercially available polymyxin B by the removal of the fatty acyl group and the terminal diamino-butyric acid residue by the proteolytic enzyme ficin following the procedure of Chihara et al., (1973) (fig 3.1). The data reported in this chapter characterises PMBN and describes its detailed analysis by a variety of chemical means. Confirmation of PMBN's lack of antibacterial activity but its ability to sensitise E.coli to some antibiotics is outlined.

A number of biophysical techniques have been used to characterise polymyxin B and PMBN. A brief description of each of these techniques is now provided.

Thin-layer chromatography is the easiest of all chromato-graphic methods for separating the products of a reaction and requires only simple and inexpensive equipment. The components of a mixture separate by migration at different rates in the mobile phase (solvent) through a cellulose alumina matrix. (e.g. see Sherma & Fried, 1984), the separated components being detected by a suitable visualisation reagent.

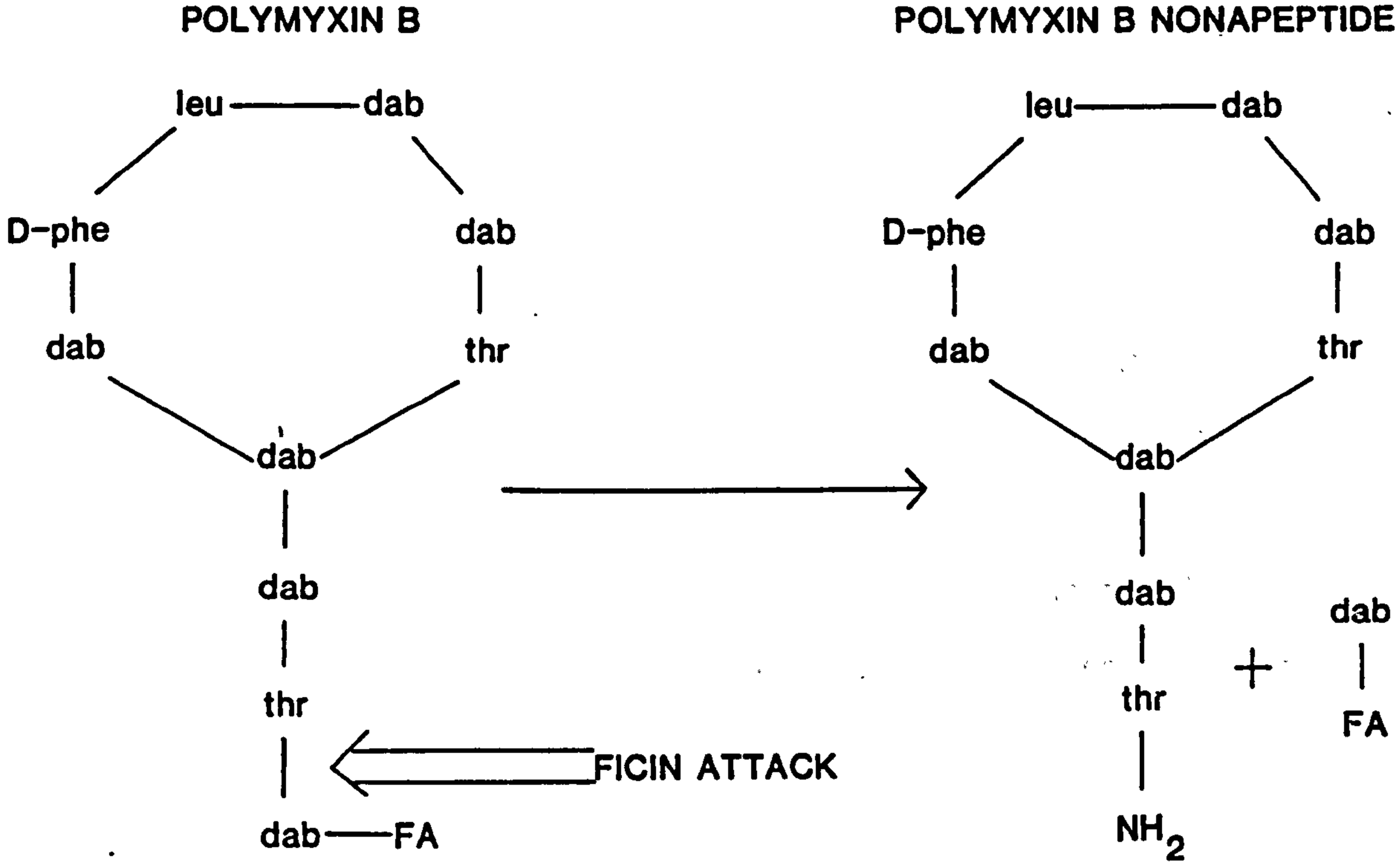


Fig 3.1 Structural formulas of polymyxin B and its derivative PMBN, prepared by enzymic ficin treatment.

Abbreviations: dab = diaminobutyric acid; phe = phenylalanine; thr = threonine; leu = leucine;

FA = fatty acyl group (6-methyl octonoic acid).

High pressure liquid chromatography is the preferred technique for improved resolutions of mixture separations. High pressure (or performance) liquid chromatography involves movement of the mixture in a high velocity mobile phase (e.g. see Engelhardt, 1979). A narrow bore column is used and is packed with particles having an average diameter of less than 50um. The efficiency of the separation depends in part on the size of column and particles.

Nuclear magnetic resonance spectroscopy has become established as a powerful technique for molecular structure elucidation. Based upon the principle of absorption and remission of radiofrequency radiation by atomic nuclei of substances placed in a strong magnetic field (e.g. see Roberts & Jardetzky, 1985). NMR spectroscopy has been used as an analytical tool to determine the precise molecular structure of many organic compounds.

Ultraviolet and visible spectroscopy; The ultraviolet or visible spectra of compounds are shown normally as a plot of the light absorbed by the compound against wavelength. Absorption spectra in the visible (400-700nm) or ultraviolet (200-400nm) are due to energy transitions of outer electrons of the molecule. Spectral analysis of such compounds involves the recording of specific absorption peaks (e.g. see Chopra, 1985a) and allows the identification and comparison of compounds of similar molecular structure (e.g. see McCormick et al., 1957).

RESULTS

3.1 PURITY AND YIELD OF PMBN

The identity and purity of the product obtained by ficin treatment of polymyxin B was checked by a number of techniques including TLC, NMR, HPLC and spectral analysis.

3.1.1 Thin-layer chromatography

Examination of TLC plates following development of spots with ninhydrin showed that the reaction product had a different R_f from that of polymyxin B or ficin (data not shown). On the basis of previous thin-layer chromatography data (Vaara & Vaara, 1983a) the product was presumed to be PMBN.

3.1.2 Spectral analysis

The spectral profiles of polymyxin B and the compound presumed to be PMBN also differed. Polymyxin B displayed four distinct absorption peaks in the 250 to 270nm range at wavelengths of 251.4, 257.4, 263.4 and 266.6nm (see fig 3.2a). The compound presumed to be PMBN was clearly related to polymyxin B because it possessed the four absorption peaks described above. However, it differed from polymyxin B by exhibiting an additional peak at 278.2nm (see fig 3.2b).

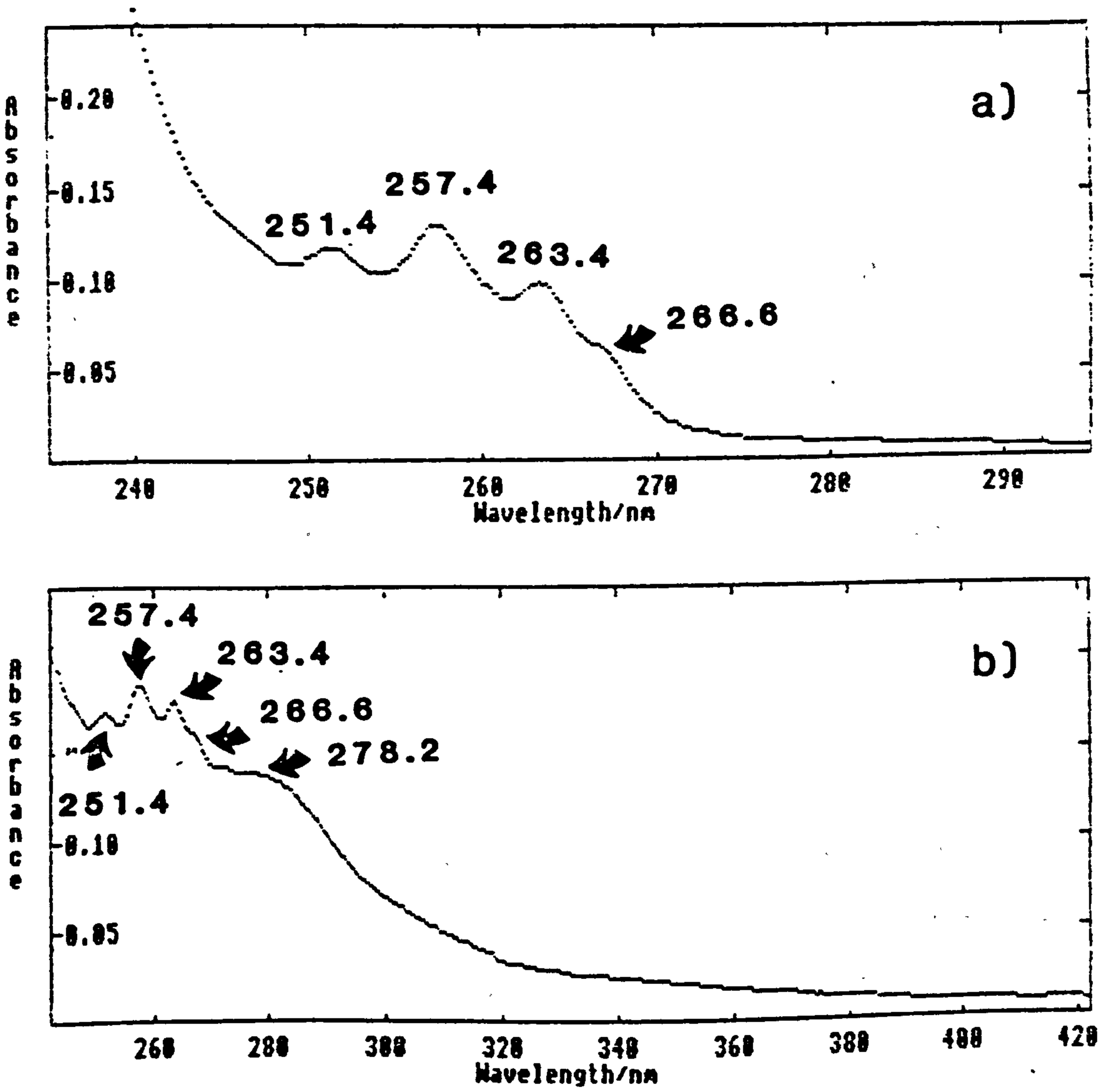


Fig 3.2 Wavelength scan profiles of (a) polymyxin B and (b) PMBN solutions obtained by transfer of data from a PU8800 spectrophotometer to a B.B.C. microcomputer (see text).

3.1.3 Nuclear magnetic resonance spectroscopy

Confirmation of the identity of PMBN was obtained by nuclear magnetic resonance spectroscopy (NMR). The signals corresponding to the terminal methyl groups and the methylene chain of the fatty acyl residue of polymyxin B were absent from the product of ficin degradation and were replaced by signals corresponding to a terminal threonine (figs 3.3 and 3.4). These results are entirely consistent with the published structure of PMBN (Vaara & Vaara, 1983c).

3.1.4 High pressure liquid chromatography

Comparison of PMBN and polymyxin B by HPLC demonstrated that they had different retention times. A solution of 100ug/ml PMBN (20ul) injected into the column produced a distinct peak almost immediately (see fig 3.5b), whereas an equivalent concentration of the polymyxin B solution produced a different profile with the retention time of the major peak delayed (fig 3.5a). Quantitative HPLC showed that batches of PMBN contained no more than 0.4% polymyxin B (data not shown). The PMBN yield was typically 70%.

3.2 PMBN STABILITY

NMR analysis of samples of PMBN which had been prepared two years previously and stored at 4^0C in dry conditions, showed that the material did not markedly deteriorate in this time (data not shown).

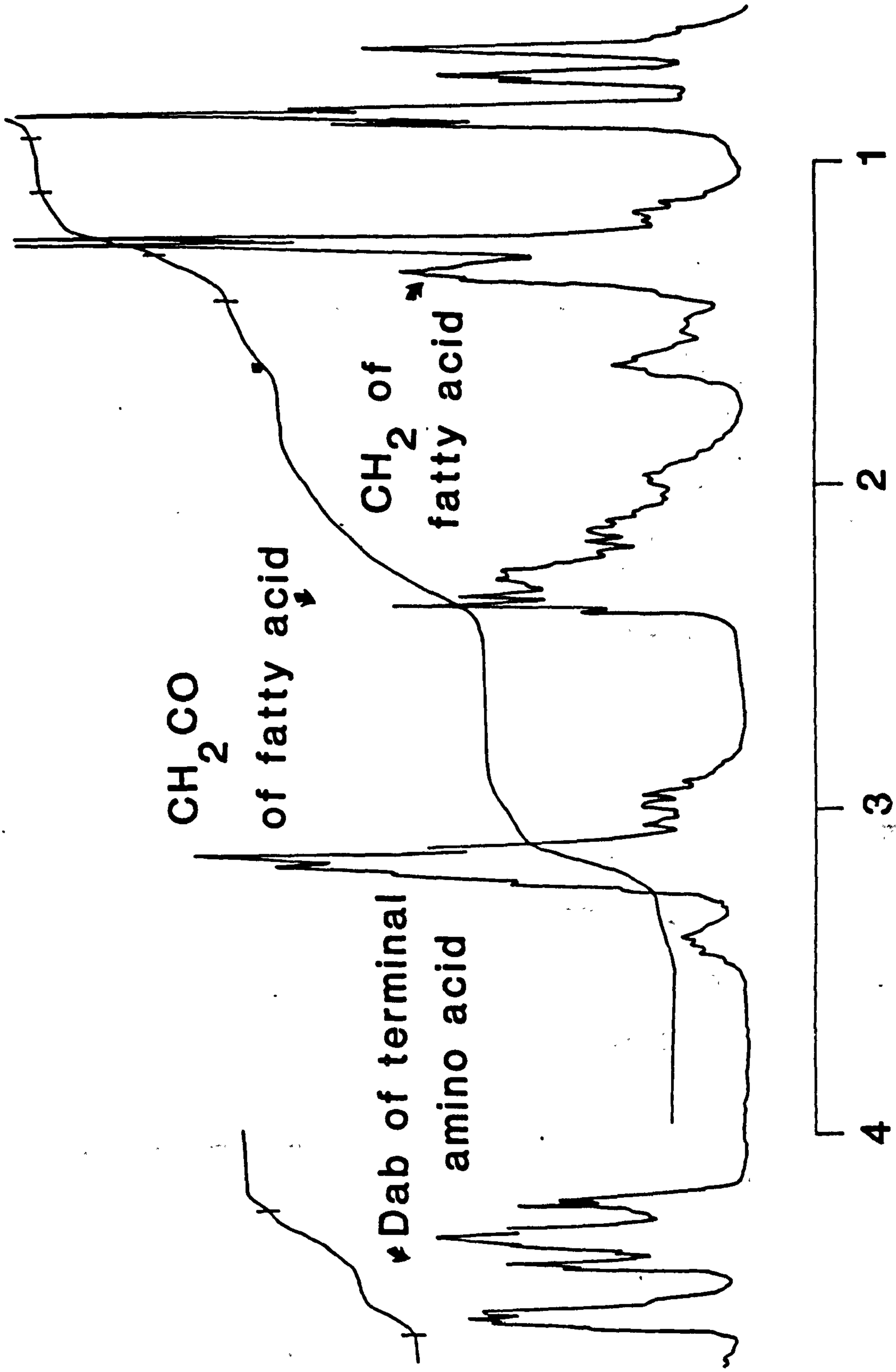


Fig 3.3 Nuclear magnetic resonance spectrum of polymyxin B.

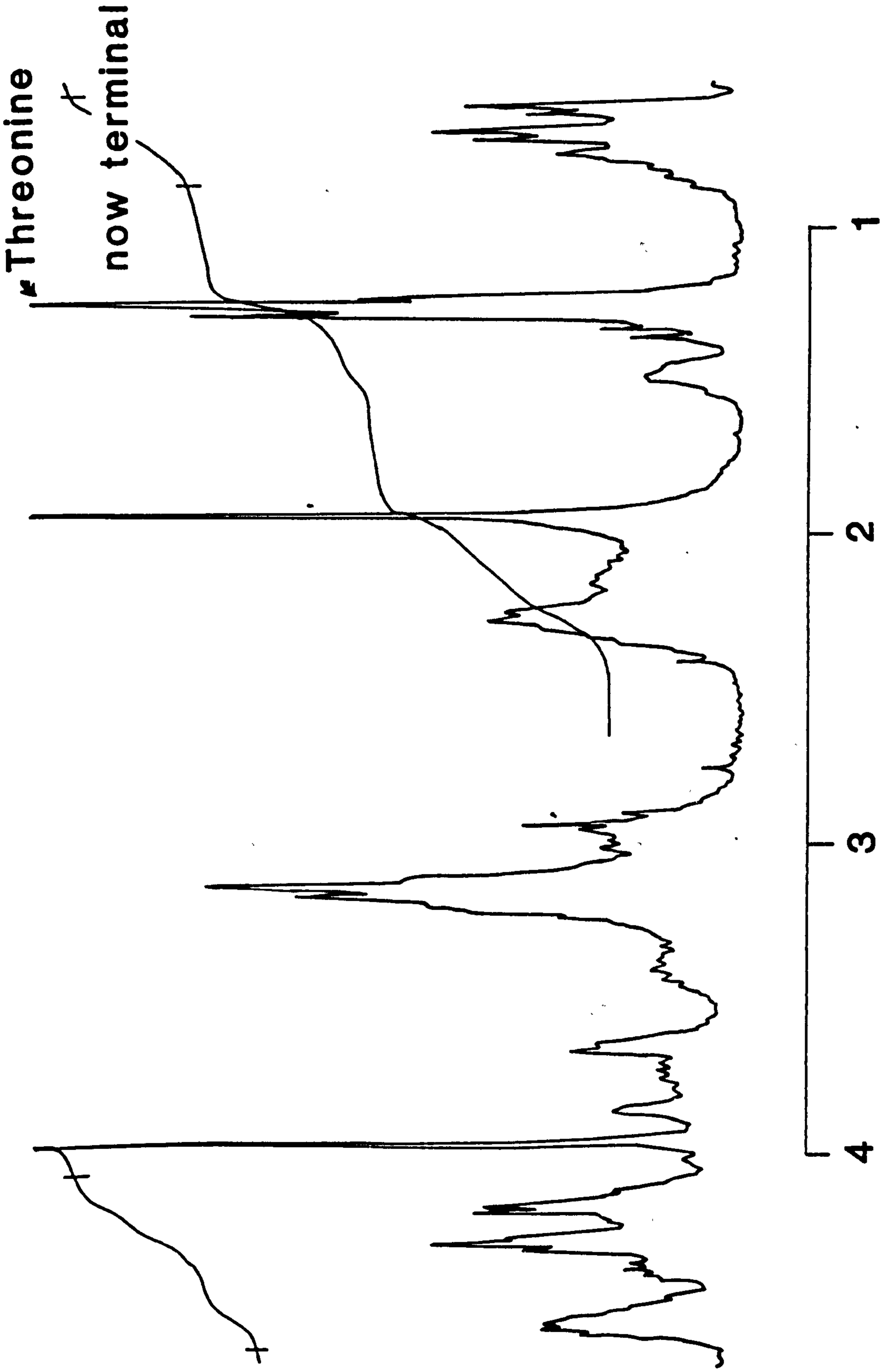


Fig 3.4 Nuclear magnetic resonance spectrum of PMBN.

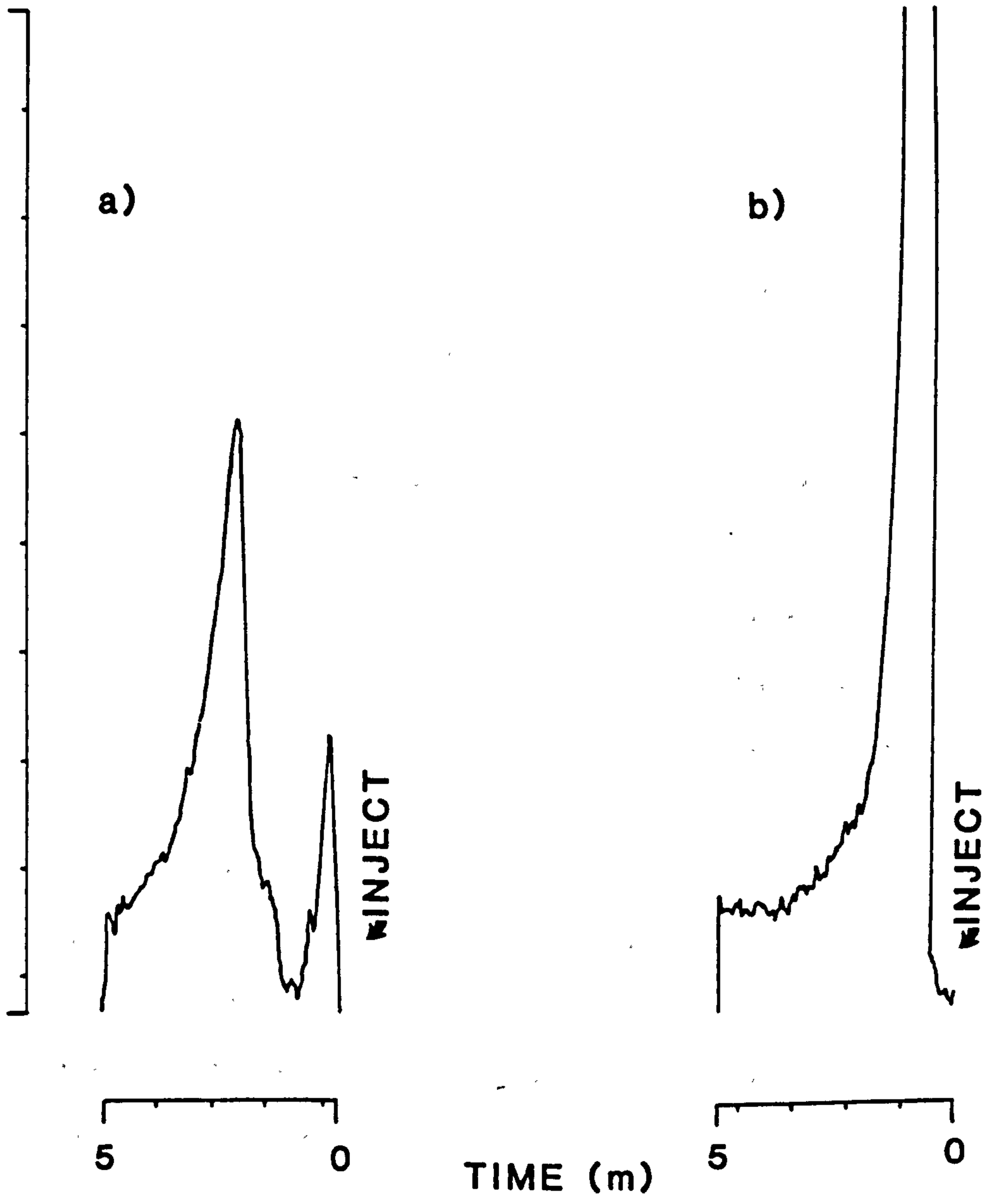


Fig 3.5 Analytical HPLC traces of (a) polymyxin B and (b) PMBN with absorbance at 210nm plotted against retention time.

3.3 THE ANTIBACTERIAL ACTIVITY OF PMBN

Compared with polymyxin B, PMBN is reported to have very low intrinsic antibacterial activity (Vaara & Vaara, 1983b and c). These findings were confirmed in the present studies by showing that the MIC value of polymyxin B for E.coli K12 3300 (pBR322) was 0.25ug/ml whereas PMBN was at least 300-fold less active (MIC >100ug/ml).

3.4 SYNERGISM BETWEEN PMBN AND HYDROPHOBIC ANTIBIOTICS

PMBN potentiates the action of hydrophobic antibiotics against Gram-negative bacteria (Alatossava et al., 1984; Vaara & Vaara, 1983c and Viljanen & Vaara, 1984). The PMBN used in these studies enhanced the activity of fusidic acid against E.coli K12 3300 thereby confirming the reports of potentiation by other workers. In a checkerboard titration of PMBN and fusidic acid (sodium salt), the MIC of fusidic acid fell from 320ug/ml in the presence of 0.5ug of PMBN per ml to 5ug/ml in the presence of 4ug of PMBN per ml (fig 3.6). Furthermore, the pattern of inhibition of growth obtained (fig 3.6) is characteristic of synergy between the two compounds (Krogstad & Moellering, 1980). Similar results were obtained with checkerboard titrations of PMBN and benzyl penicillin (data not shown). The results obtained with PMBN were not simply due to residual contamination with polymyxin B, because checkerboard titrations with polymyxin B in the range 2-32 ng/ml (representing the upper limit of contamination of PMBN

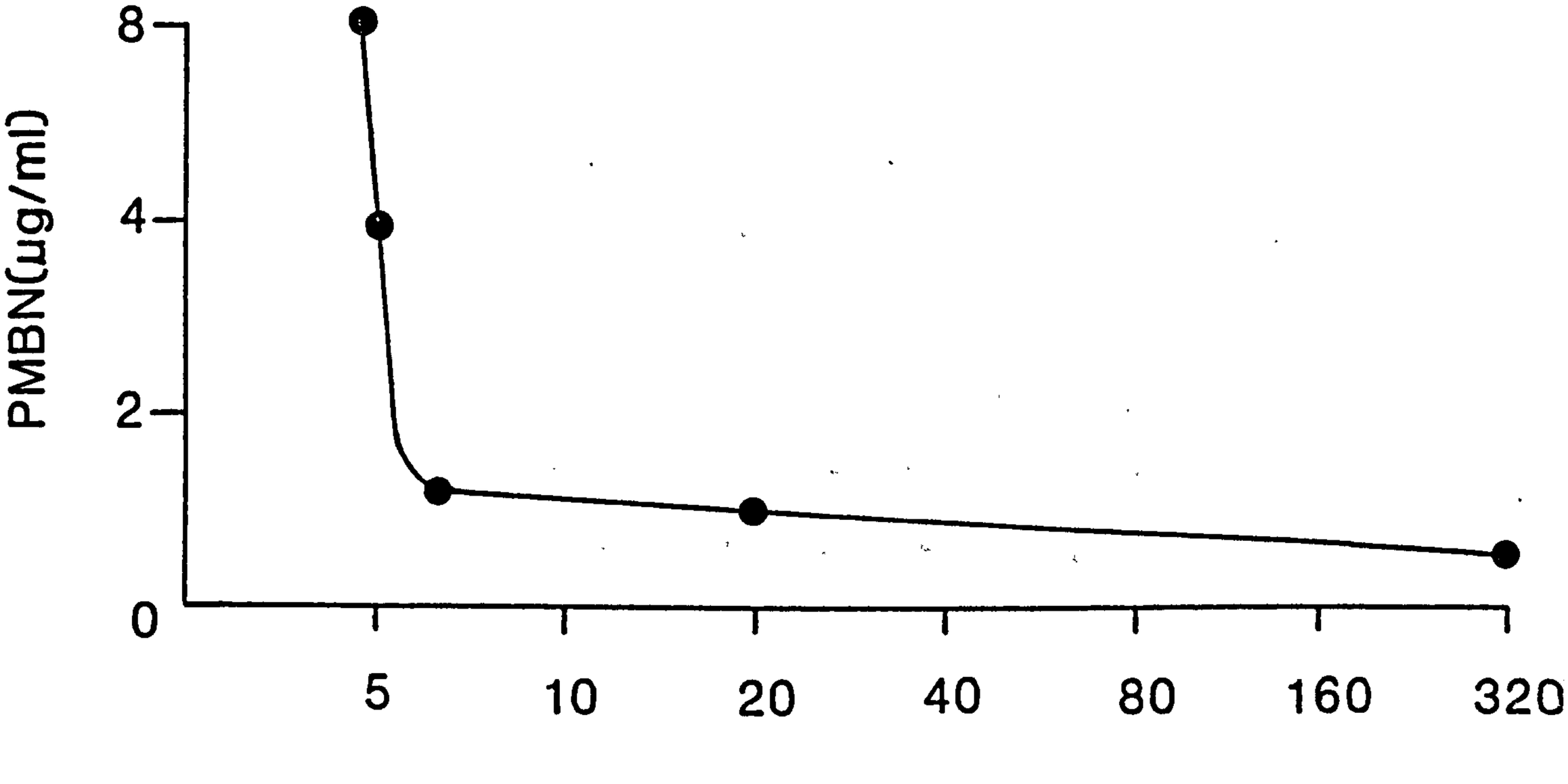


Fig 3.6 Minimum inhibitory concentrations of sodium fusidate (ug/ml) against E.coli K12 3300 (pBR322) in the presence of PMBN (0.5-8ug/ml). Graphical representation of typical results obtained in a checkerboard titration of sodium fusidate and PMBN.

with polymyxin B, i.e. 0.4%) failed to decrease the fusidic acid or benzyl penicillin MIC value (data not shown).

3.5 VIABILITY OF E.COLI K12 3300 (pBR322) AFTER EXPOSURE TO POLYMYXIN B OR PMBN

Viability determinations following exposure to polymyxin B or PMBN were performed on E.coli K12 3300 (pBR322) suspended at cell densities of 10^{10} cells per ml in 10mM sodium phosphate buffer (pH 7.4). Treatment with 200ug/ml of polymyxin B led to rapid bacteriolysis (fig 3.7) whereas an equivalent concentration of PMBN had little or no killing effect.

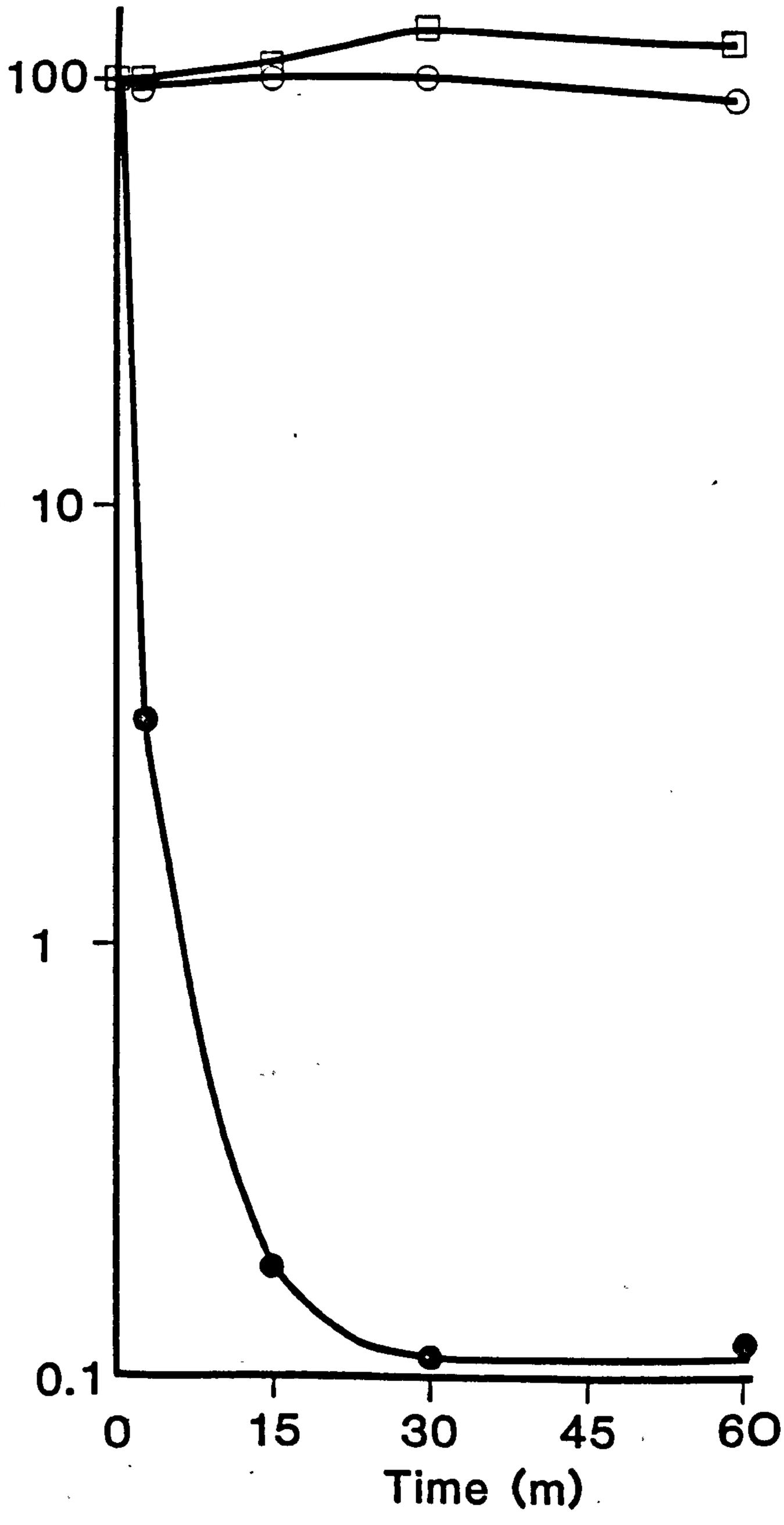


Fig 3.7 Viability of E.coli K12 3300 (pBR322) after exposure to polymyxin B or PMBN: Polymyxin B (●) 200ug/ml; PMBN (O) 200ug/ml; Untreated controls (□). Values are the means of replicate determinations.

DISCUSSION

Chemical analysis of PMBN preparations confirmed the reported structure of PMBN. Spectral analysis of both PMBN and polymyxin B showed that PMBN could be distinguished from its parent by characteristic absorption spectra. In addition, the application of NMR spectroscopy has provided the first direct evidence that the structure of PMBN is as predicted by Vaara & Vaara, (1983a), and that the compound exhibits a terminal threonine residue in place of the terminal diaminobutyric acid (dab) residue and fatty acyl chain removed by ficin. Vaara & Vaara, (1983a and b) omitted to report any physical methods of analysis to elucidate the structure of PMBN.

PMBN prepared for this study has been subjected to considerable purity analysis. Previous reports by Vaara & Vaara, (1983a and b) have described the TLC analysis of ficin-treated polymyxin B demonstrating the product PMBN and polymyxin B as separate spots. This present study has confirmed the findings of these workers but in addition has extended the purity analysis of PMBN using HPLC, a superior high resolution technique to TLC.

To study PMBN action it has been important to exclude the possibility that changes affecting permeability of the envelope were not due simply to low levels of contaminating polymyxin B. As previously outlined the outer membrane of Gram-negative bacteria is essentially the primary target for disruption by polymyxin B and its integrity may well be affected by very low

levels of the antibiotic. Indeed, sublethal concentrations of polymyxin B (0.3ug/ml) have been reported as responsible for the slight sensitisation of E.coli to fusidic acid (Vaara & Vaara, 1983a). Investigations of the purity of PMBN by HPLC techniques have given a quantitative estimate of organic contamination, especially with respect to polymyxin B. The extent of polymyxin B contamination of PMBN preparations was very low (<0.4%). Residual polymyxin B contamination is therefore unlikely to account for changes in permeability induced by PMBN preparations.

The microbiological results presented in this chapter have also confirmed 1) some of the fundamental features of PMBN action, i.e. the ability of PMBN to sensitise polymyxin -susceptible organisms such as E.coli to various hydrophobic antibiotics (Alatossava et al., 1984; Vaara & Vaara, 1983c; Viljanen & Vaara, 1984; and Lam et al., 1986) 2) the poor antibacterial activity of PMBN against E.coli compared to the parent compound polymyxin B (Vaara & Vaara, 1983a and b) 3) that relatively high concentrations of PMBN failed to kill cells whereas polymyxin B was highly bacteriolytic (Vaara & Vaara, 1983b). It was shown, however, that although PMBN failed to kill cells during treatment, a number of biochemical parameters (detailed in later chapters) were altered.

CHAPTER 4

EFFECTS OF POLYMYXIN B NONAPEPTIDE AND POLYMYXIN B ON THE CELL ENVELOPE STUDIED BY PROTEIN LEAKAGE

INTRODUCTION

The previous chapter described the characterisation of PMBN and the initial experiments that confirmed its ability to sensitise E.coli to some hydrophobic antibiotics without killing the cell. This chapter deals with aspects of the interaction of PMBN with the E.coli cell envelope.

The outer membrane of Gram-negative organisms acts as a permeability barrier to macromolecules including hydrophobic antibiotics (Nikaido & Nakae, 1979). Although the mechanism leading to the loss of the barrier function to hydrophobic antibiotics is unknown, it would seem reasonable to suppose that some degree of perturbation of the outer membrane has taken place, and this may be reflected by the leakage of proteins from the periplasmic region. Several approaches have been taken to investigate the outer membrane as a functional barrier. Firstly, exposure of cells to EDTA increases the permeability, by chemical means, of a wide range of Gram-negative bacteria to many compounds (Hancock, 1984). Treatment of E.coli with EDTA enhances passage of hydrophobic antibiotics through the cell envelope (Leive, 1974) resulting in concomitant loss of periplasmic proteins through the damaged outer membrane (Cerny & Teuber, 1972). Secondly, deep rough lipopolysaccharide mutants that are hypersensitive to hydrophobic antibiotics also release periplasmic proteins into the medium (Nikaido & Vaara, 1985). These general features are illustrated in fig 4.1. Treatment of E.coli with PMBN, however, does not apparently cause leakage of

Fig 4.1 Hypothetical structure of outer membrane in the wild-type, deep rough mutants and EDTA-treated wild-type cells of E.coli.

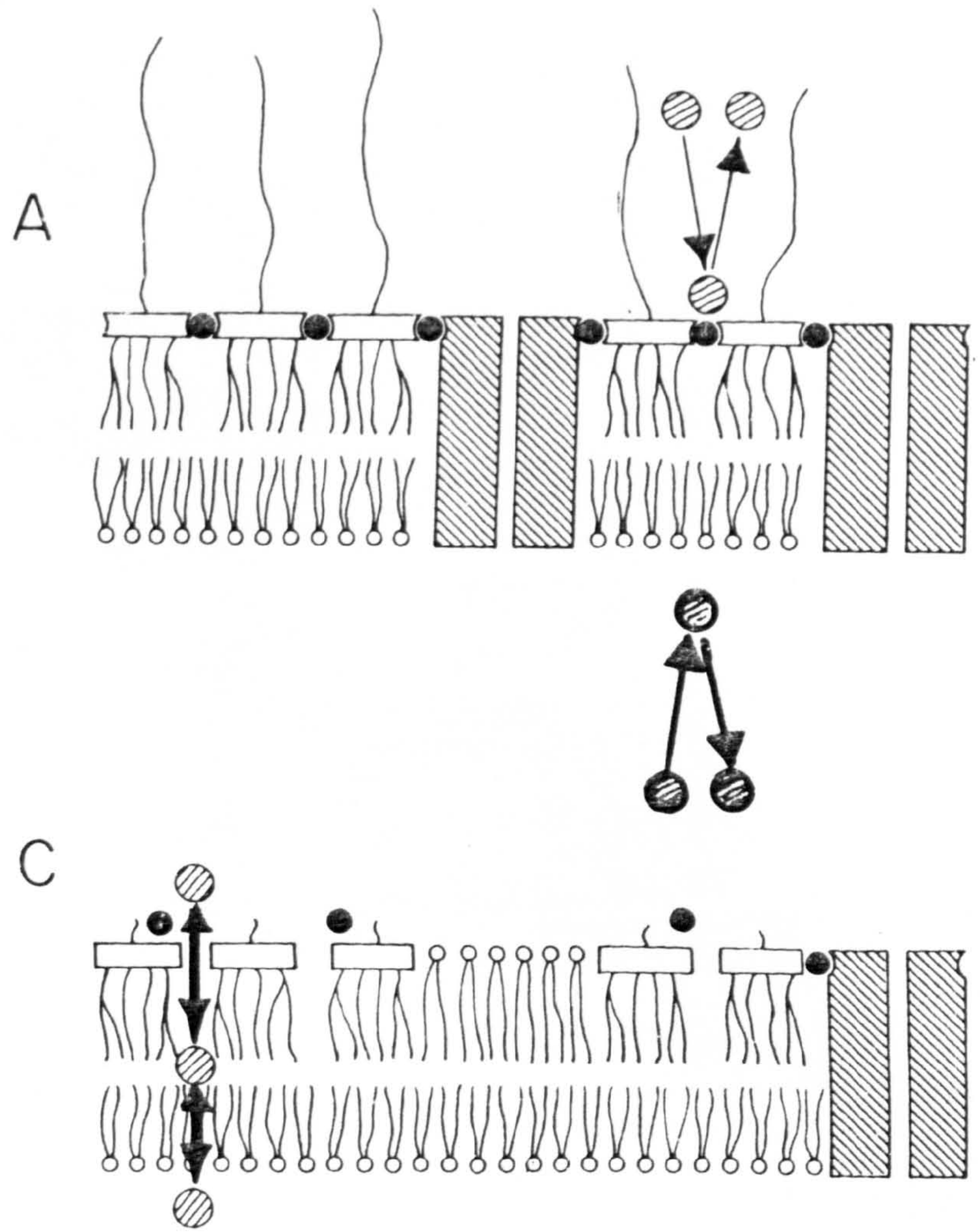
A) Wild-type strain in which the outer layer is almost entirely composed of LPS and protein. Periplasmic proteins (⊘) are restricted to the cell.

B) Deep rough mutants. Hydrophobic molecules (⊘) are assumed to penetrate through the phospholipid bilayer domains. Periplasmic proteins can apparently leak through this area.

C) Deep rough mutants. Hydrophobic molecules are assumed to penetrate through the LPS domains which become more permeable due to alterations in LPS structure. Periplasmic proteins can apparently leak through this area.

D) EDTA-treated wild-type cells. Phospholipid molecules are assumed to fill the void left by the selective removal of LPS by EDTA. Small amounts of periplasmic proteins leak after this treatment.

(modified from Nikaido & Vaara 1986).


A
B
LPS
Mg^{2+} or Ca^{2+}
PHOSPHOLIPID
PROTEIN
C
D

proteins from the cell (Vaara & Vaara, 1983b and Nikaido & Vaara, 1985). This would seem to be an unusual feature since the compound can enhance uptake of hydrophobic antibiotics. Despite reports to the contrary (Vaara & Vaara, 1983b and Nikaido & Vaara, 1985) PMBN may indeed cause loss of periplasmic proteins from Gram-negative bacteria accompanied by changes in the permeability of the outer membrane to hydrophobic antibiotics.

In this chapter the possibility that PMBN promotes periplasmic protein leakage in E.coli has been re-examined. The activity of PMBN in this respect was compared directly with that of polymyxin B, which is known to cause release of periplasmic proteins (Cerny & Teuber, 1971 and 1972; Storm et al., 1977 and Vaara & Vaara, 1981).

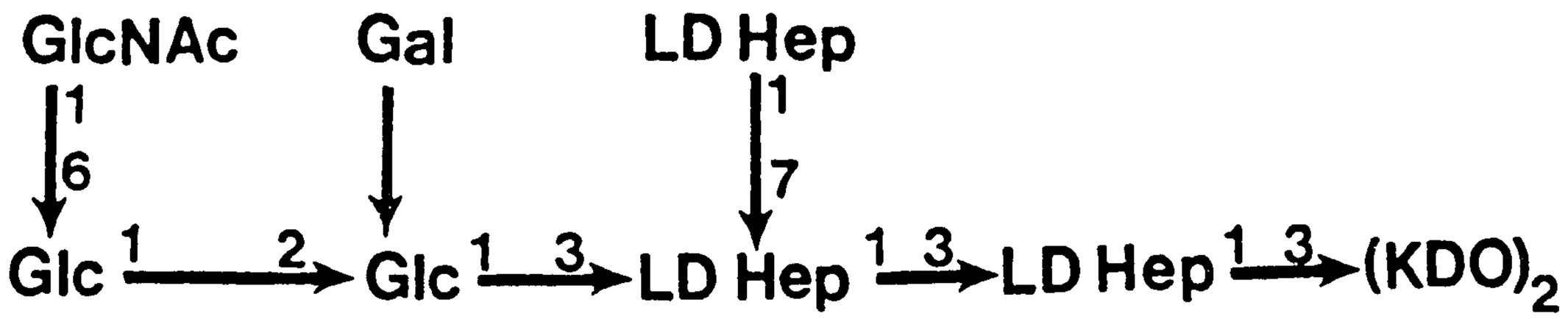


Fig 4.2 Core region of E.coli K12 lipopolysaccharide (from Hammond et al 1984).

RESULTS

4.1 POLYMYXIN B AND PMBN-INDUCED RELEASE OF MACROMOLECULES FROM E.COLI

4.1.1 Lipopolysaccharide

It is well established that KDO is a component of E.coli LPS (fig 4.2). Possible loss of LPS following treatment of bacteria with polymyxins was assessed by assaying for KDO in cell-free supernatants (Karkhanis et al., 1978). There was no evidence that these compounds promoted release of LPS (data not shown).

4.1.2 Proteins; the kinetics of leakage and cell compartments from which proteins are lost.

Concentrated suspensions of E.coli K12 3300 (pBR322) were exposed to polymyxin B (200ug/ml) or PMBN (200ug/ml), and the release of protein was determined from cell-free supernatants at various times after exposure to the compounds. Polymyxin B caused release of about 100ug/ml of protein per ml of supernatant after 1 min, reaching a maximum of about 180ug/ml after 45 min (fig 4.3). PMBN also caused loss of protein within the first minute. However, the quantity of protein released was less than that from polymyxin B-treated cells (fig. 4.3). Untreated bacteria failed to release detectable quantities of protein (fig 4.3).

To examine whether released protein originated from either the periplasmic space or the cytoplasm, the supernatants were

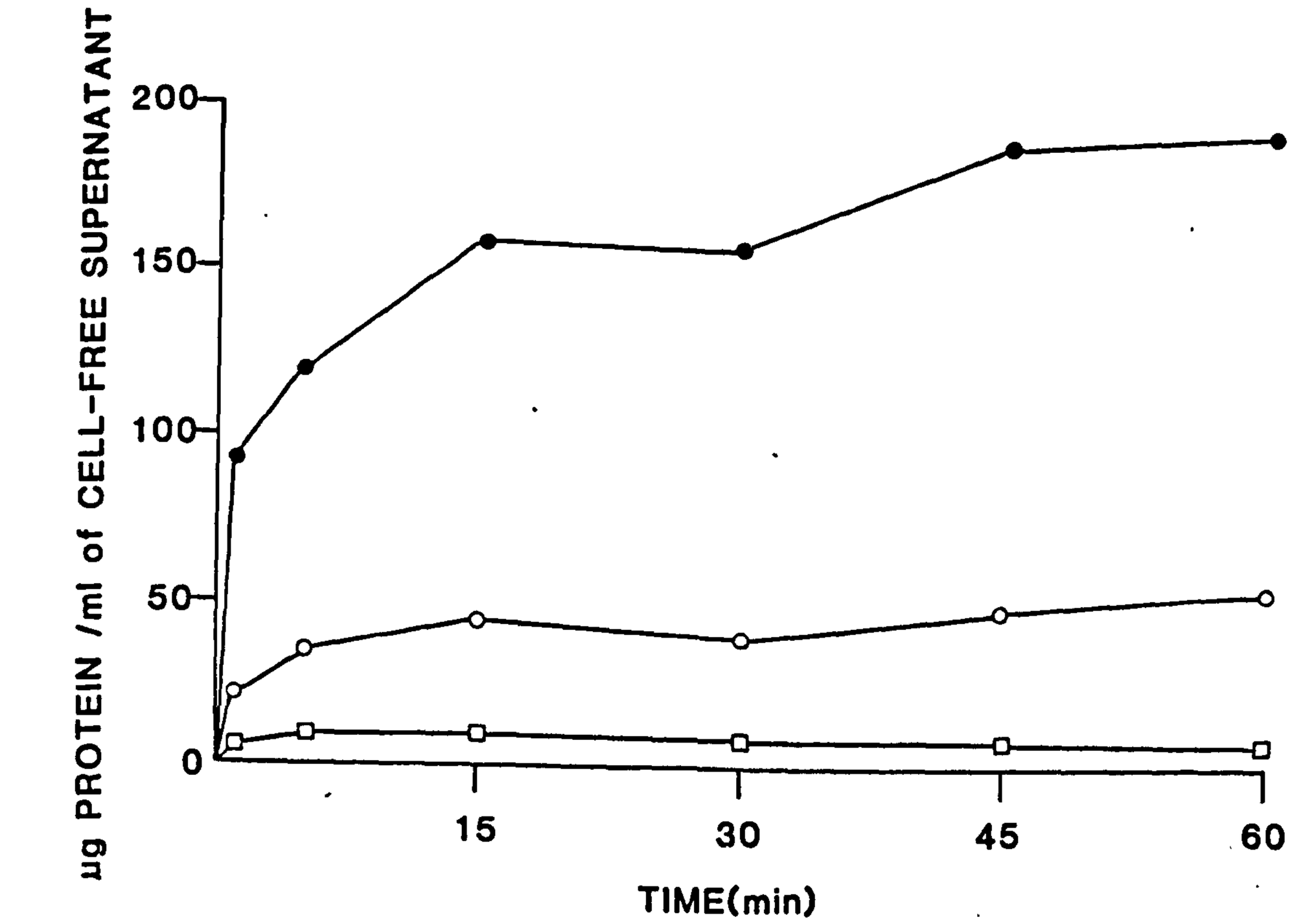


Fig 4.3 Polymyxin B and PMBN-mediated release of protein from E.coli K12 3300 (pBR322). Cell suspensions were divided into three samples. At time zero polymyxin B (200ug/ml) was added to one portion (●) and PMBN (200ug/ml) (O) to the second. The third sample served as an untreated control (□). Bacteria were incubated at 37^0C and samples removed at the times indicated. Samples were processed rapidly to remove bacteria, followed by estimation of protein in the cell-free supernatant. Values are the means of duplicate determinations.

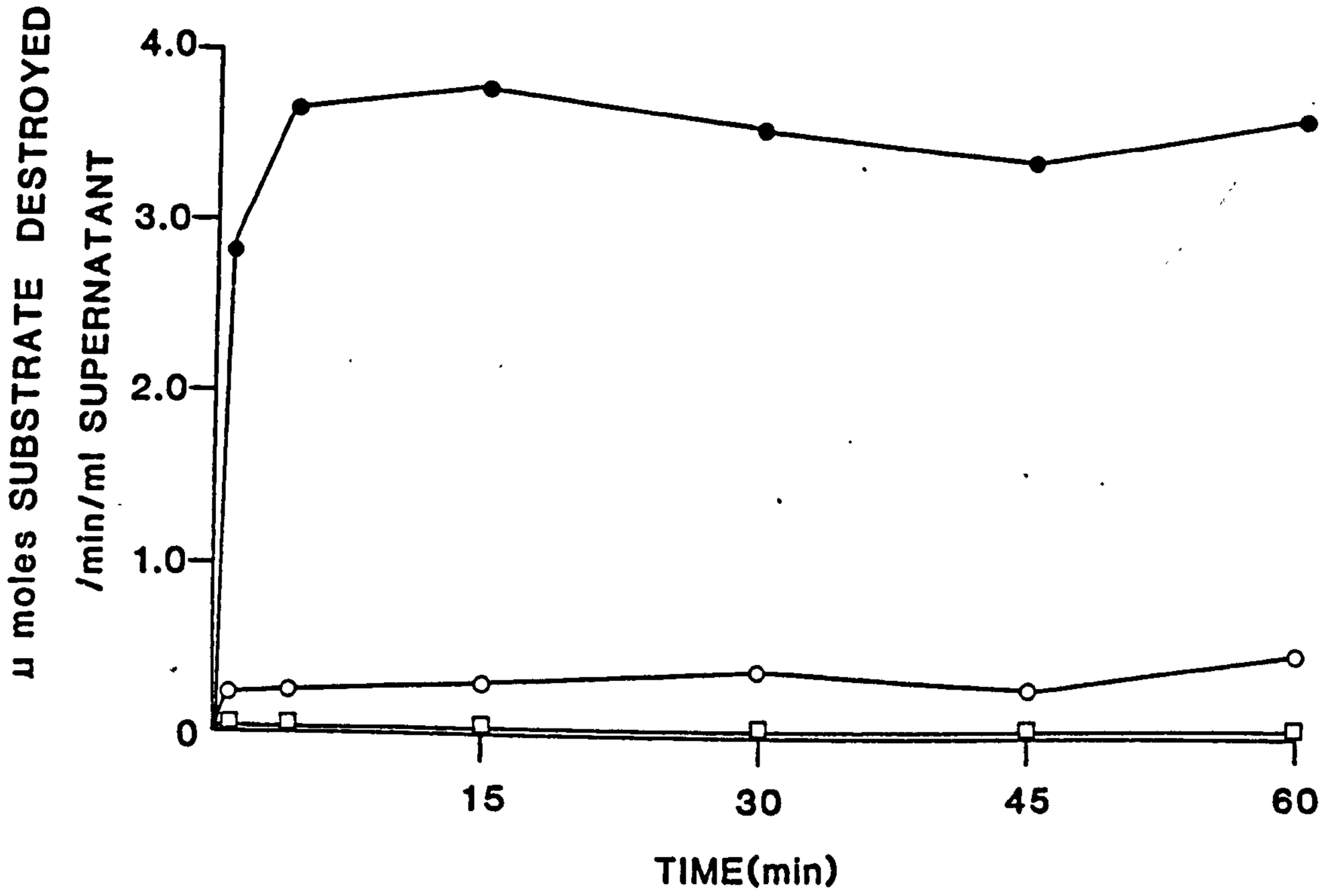


Fig 4.4 Polymyxin B and PMBN-mediated release of beta-lactamase from E.coli K12 3300 (pBR322).

The experiment was conducted as described in the legend to fig 4.3 except that the cell-free supernatants were assayed for beta-lactamase activity: Polymyxin B (●) 200ug/ml; PMBN (○) 200ug/ml; Untreated control (□). Values are the means of duplicate determinations.

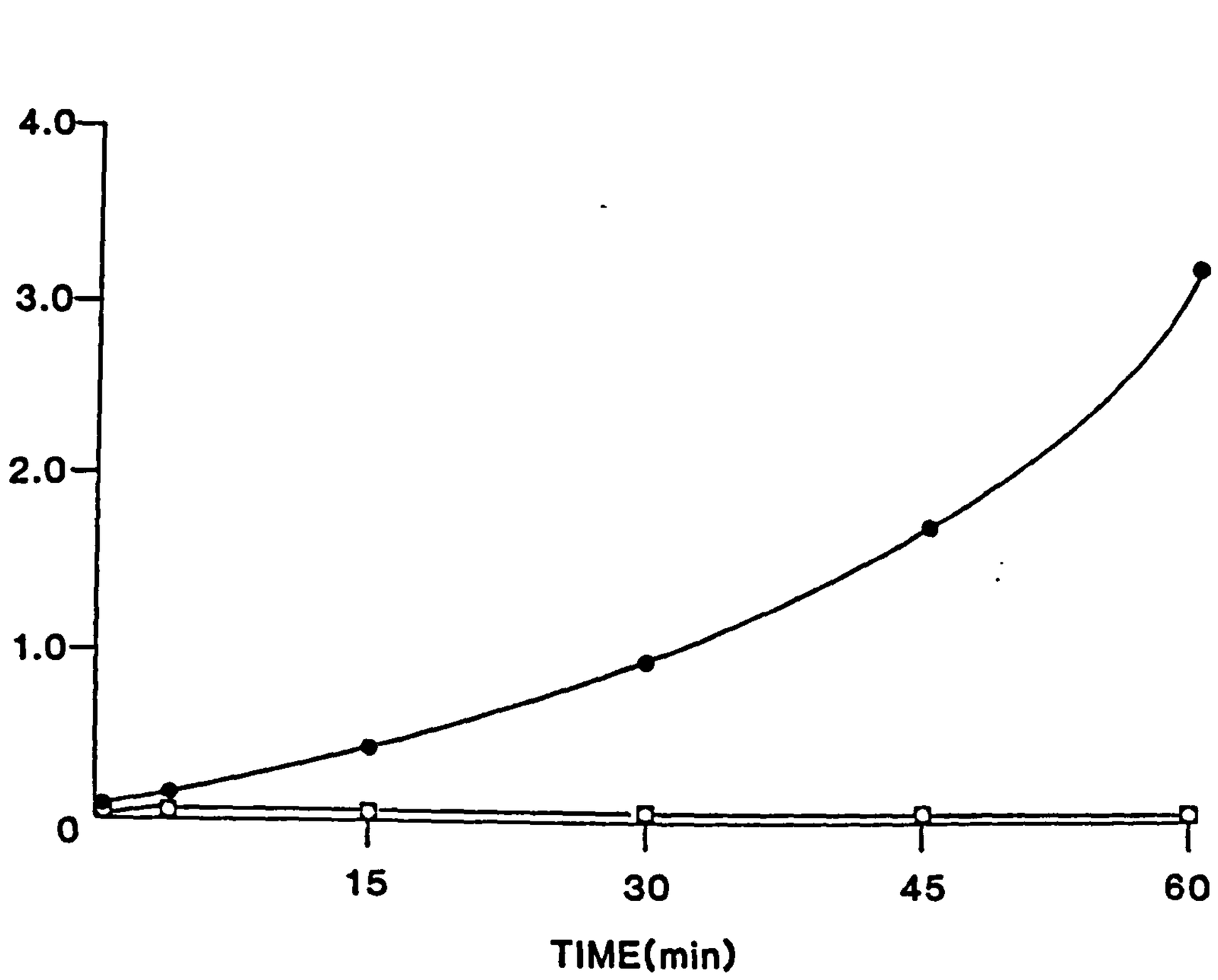


Fig 4.5 Polymyxin B-mediated release of beta-galactosidase from E.coli K12 3300 (pBR322). The experiment was conducted as described in the legend to fig 4.3 except that the cell-free supernatants were assayed for beta-galactosidase activity: Polymyxin B (●) 200ug/ml; PMBN (○) 200ug/ml; Untreated control (□). Values are the means of duplicate determinations. ONP = o-nitrophenyl-B-D-galactopyranoside.

assayed for beta-lactamase (periplasmic) and beta-galactosidase (cytoplasmic). Polymyxin B treatment caused immediate release of beta-lactamase which was complete within 5 min of exposure to the antibiotic (fig 4.4). After 60 min this represented release of 62% of the cellular beta-lactamase activity. An equivalent concentration of PMBN (200ug/ml) also caused beta-lactamase release (fig 4.4). The kinetics of polymyxin B and PMBN-mediated release were similar, but the level of enzym released by PMBN was about seven-fold lower than that liberate by polymyxin B (fig 4.4). Untreated bacteria also showed no release of beta-lactamase. The results obtained with PMBN wer not due simply to contamination with polymyxin B because exposure of bacteria to 0.8ug/ml of polymyxin B (representing the maximum level of contamination of PMBN with polymyxin B) caused no detectable protein or beta-lactamase release.

Polymyxin B also caused release of beta-galactosidase int the supernatant, although release of the enzyme was not detected until the bacteria had been treated with polymyxin B for 15 min (fig 4.5). In contrast, PMBN-treated bacteria failed to release beta-galactosidase even after treatment for 60 min and in this respect their behaviour was similar to that of untreated bacteria from which there was also no release of enzyme (fig 4.5).

4.2 PROTEIN LEAKAGE : ANALYSIS BY GEL ELECTROPHORESIS

The results described in the previous section suggest that among the proteins released by PMBN, at least some are likely

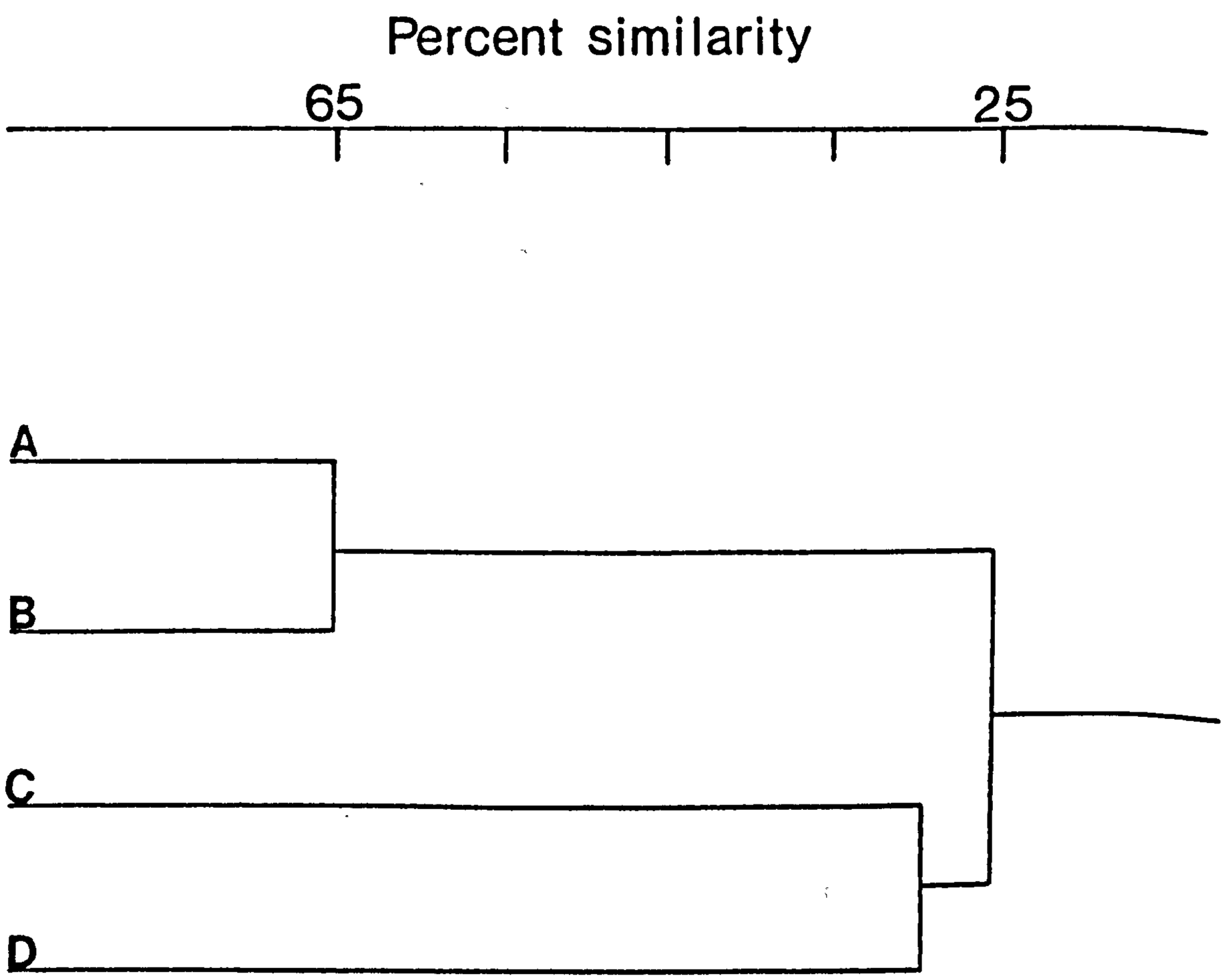


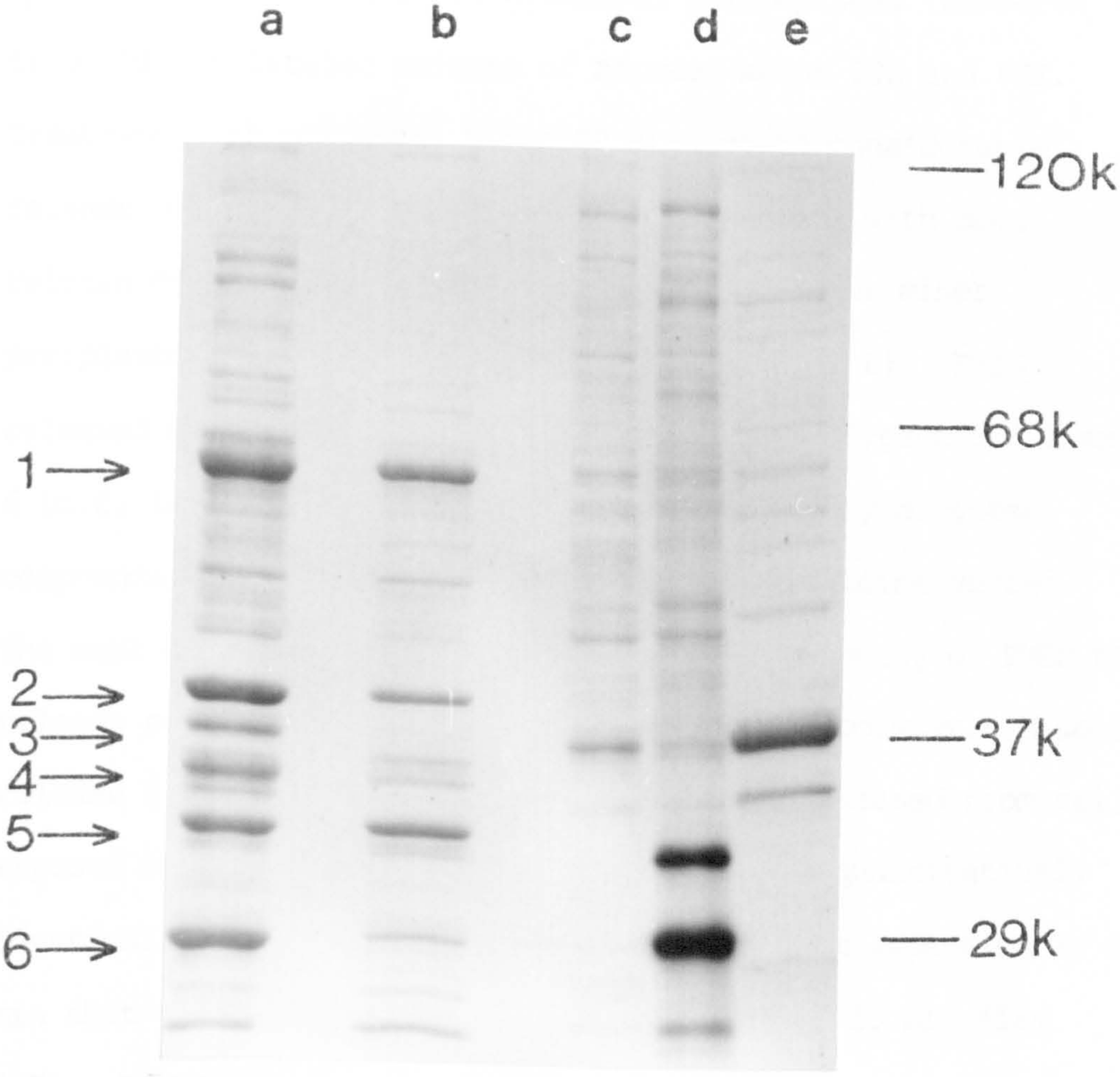
Table 4.1 Comparison of polypeptide profiles by single linkage clustering analysis. The presence or absence of 31 individual peaks from each gel profile was recorded. Percentage similarity of the profiles was calculated by computer program and shown above as a dendrogram. Comparisons of the following fractions from E.coli K12 3300 (pBR322) are shown:- A) periplasmic proteins; B) polymyxin-released proteins; C) PMBN-released proteins (as shown in figs 4.6 and 4.7) and D) outer membrane.

to be derived from the periplasmic region. Some of the proteins released by polymyxin B are also likely to be periplasmic, but in addition cytoplasmic proteins are probably released by exposure to the antibiotic. To confirm these suggestions and also to establish whether outer membrane or cytoplasmic membrane proteins might also be released by either of the compounds, further experiments were conducted in which released proteins were analysed by gel electrophoresis and the profiles compared with those obtained from separately prepared cell fractions.

Proteins from the cytoplasmic (inner) membrane, periplasmic and outer membrane fractions were compared with those released by polymyxin B or PMBN after exposure to the compounds for 60 min. The degree of similarity between the various preparations was estimated on the basis of both polypeptide migration measurements (obtained with the Joyce Loebl scanning densitometer) and the overall profiles exhibited by each sample. The degree of similarity of protein profiles from various cell fractions was further assessed by single linkage clustering analysis. The polypeptides released by polymyxin B (after 60 min) showed a high degree of similarity with periplasmic proteins (fig 4.6) and less resemblance to proteins contained in the other cell fractions (data not shown). This was consistent with the findings of Cerny & Teuber (1971 and 1972) and was confirmed by clustering analysis shown in table 4.1.

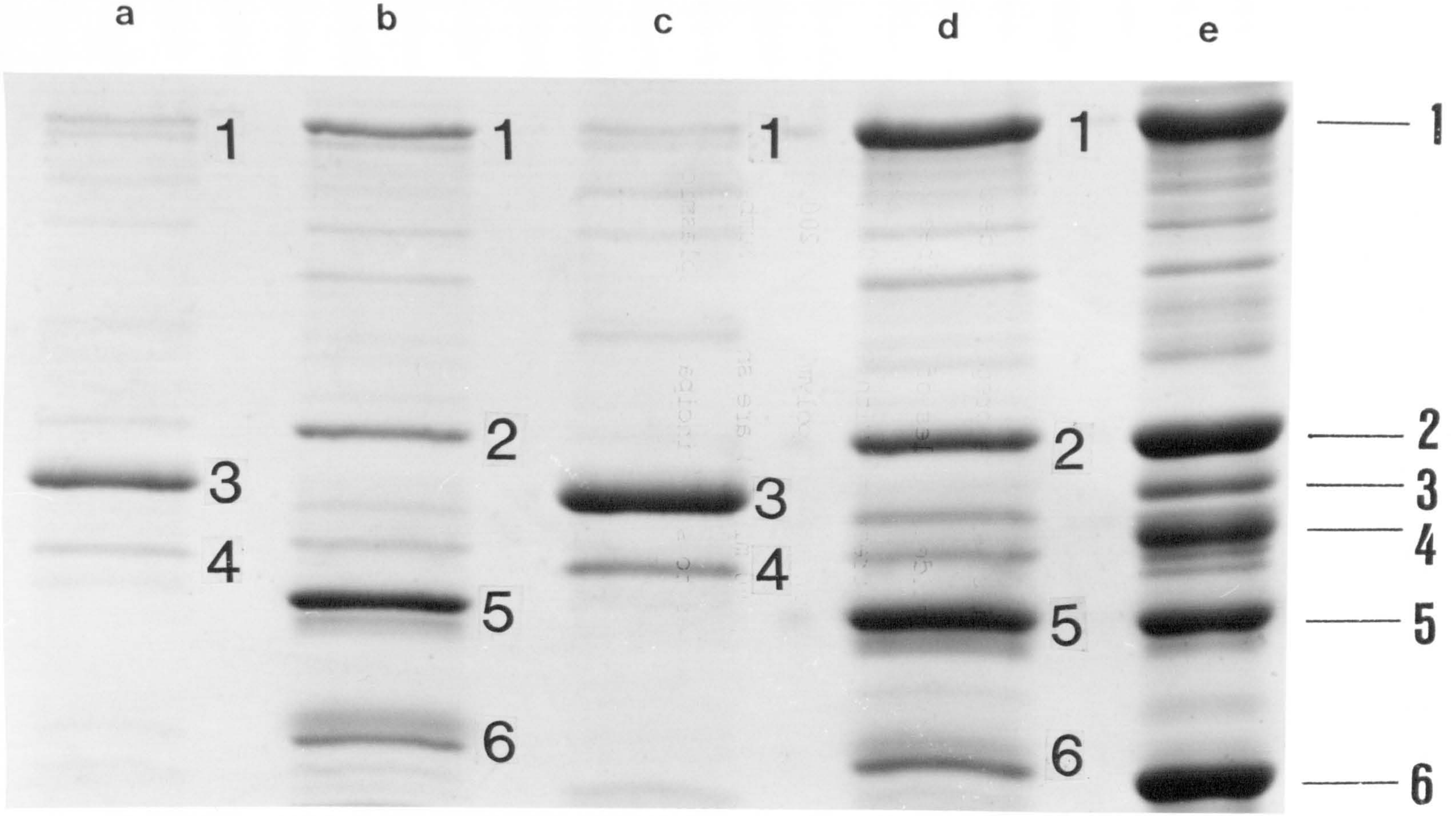
Proteins released by PMBN (after 60 min) also appeared to be periplasmic (fig 4.6) which was further confirmed by showing

Fig 4.6 Comparison of polypeptides released by polymyxin B (lane b) and PMBN (lane e) with periplasmic proteins (lane a) and pBR322-encoded proteins synthesized in minicells (lane d). E.coli K12 3300 (pBR322) cells (10^{10}/ml of phosphate buffer) were treated with polymyxin B (200ug/ml) or PMBN (200ug/ml) for 60 min. Proteins released into cell-free supernatants were electrophoresed. Periplasmic proteins were also prepared (see text) and separated (lane a). Minicells containing pBR322 were labelled with [^{35}S]-methionine and coelectrophoresed (lane c) with the other samples. After electrophoresis proteins in lane a, b, c, and e were detected with Coomassie blue. The gel was then dried, and labelled polypeptides in lane c were detected by autoradiography. The X-ray film was aligned over the dried stained gel to locate lane d (labelled proteins) between lanes c and e. A composite photograph was taken to provide the figure shown. Numbers 1 - 6 on the left refer to six principal polypeptides (see text). Molecular weights are shown on the right.


a
b
c
d
e
120k
68k
37k
29k
1
2
3
4
5
6

that the profiles had little resemblance to the other cell fractions (table 4.1). However, PMBN clearly differed from polymyxin B with respect to the release of individual periplasmic polypeptides (fig 4.6). The periplasmic fraction (lane a fig 4.6) prepared by the lysozyme/EDTA method of Yamato et al (1975) contained six principal polypeptides (numbered 1 to 6) with molecular weights of between about 29K and 62K. Treatment with polymyxin B for 60 min led to considerable release of periplasmic polypeptides 1, 2 and 5 with some release of polypeptides 4 and 6 as well as other minor periplasmic proteins (c.f. lanes a and b fig 4.6). PMBN released polypeptides 3 and 4 and traces of polypeptides 1 and 6 (c.f. lane e fig 4.6). Therefore, the ability of these compounds to release the major periplasmic proteins varied. The most characteristic features being the capacity of PMBN to release polypeptide 3 but not 2 and 5 whereas polymyxin B could release 2 and 5 but not 3. The polypeptides released from cells exposed to polymyxin B for 15 and 60 min were quantitatively identical, with no evidence of additional bands appearing at 60 min that were absent from the samples taken at 15 min (fig 4.7). The poly- peptide profiles of supernatants taken after 15 and 60 min exposures to PMBN were also quantitatively identical (fig 4.7). Furthermore, the ability of PMBN to promote the release of similar polypeptides to those above, from other strains of E.coli was noted. E.coli K12 DC0 described by Curtis et al., 1979a and b) produced a comparable polypeptide profile of released polypeptides to that of E.coli K12 3300 (pBR322) (data not shown).

Fig 4.7 Comparison of polypeptides released following treatment with polymyxin B or PMBN after 15 or 60 min. E.coli K12 3300 (pBR322) was treated and prepared as described in the legend to fig 4.6. Periplasmic proteins (e) were prepared as described in the text. The polypeptide profiles of cell-free supernatants after treatment with PMBN (200ug/ml) for 15 min (a) or 60 min (c); or polymyxin B (200ug/ml) for 15 min (b) or 60 min (d) are shown. Numbers 1 - 6 on the right refer to six principal periplasmic polypeptides.


a
b
c
d
e
1
2
3
4
5
6

4.3 PARTIAL CHARACTERISATION OF PROTEINS RELEASED BY POLYMYXIN B and PMBN

The active form of pBR322-encoded beta-lactamase migrates as a 28,500 (28.5K) molecular weight polypeptide in polyacrylamide gels (Kopylova-Sviridova et al., 1979). Periplasmic polypeptide 6 (figs 4.6 and 4.7) therefore probably represents beta-lactamase. To confirm this, minicells from DS410 (pBR322) were prepared and plasmid-encoded proteins labelled with [^{35}S] methionine. Labelled preparations were co-electrophoresed with non-labelled periplasmic proteins together with preparations obtained after treatment with polymyxin B and PMBN (fig 4.6). The major labelled products in minicells containing pBR322 were 28.5K and 34K proteins, corresponding respectively to the pBR322-encoded beta-lactamase and the cytoplasmic membrane protein responsible for tetracycline efflux (Kopylova-Sviridova et al., 1979 and Chopra, 1985a). Periplasmic polypeptide 6 (lane a fig 4.6) co-migrated with the labelled beta-lactamase synthesised in minicells (lane d fig 4.6). As noted above periplasmic polypeptide 6 was released by polymyxin B, whereas only trace amounts of this polypeptide were released by PMBN (the level of polypeptide 6 released by PMBN is too low to be reproduced in lane e of fig 4.6). These observations are also consistent with the identification of polypeptide 6 as beta-lactamase because polymyxin B released more of this enzyme than did PMBN (fig 4.4). PMBN failed to release detectable quantities of 34K

polypeptides (fig 4.6), indicating that the pBR322-encoded tetracycline efflux protein was not released from the cytoplasmic membrane.

In conventional PAGE, the OmpF/C complex (porins) is difficult to resolve and appears normally as a single band. However, separation of the major outer membrane proteins of an E.coli strain (wild-type K12 described by Henning et al., 1978) was achieved with ultra-thin (1mm) gradient gels. OmpF and OmpC produced two separate bands and OmpA was also resolved (lane a fig 4.8). Polypeptide 3 (c.f. lane e fig 4.6) was released by PMBN from this strain and co-migrated with OmpF rather than either OmpC or OmpA (lane b fig 4.8). The possibility therefore remains that some of the proteins released by PMBN, and in particular polypeptide 3, may represent OmpF (fig 4.8). Thus various experiments were conducted to establish whether or not polypeptide 3 is an outer membrane protein.

4.4 CONFIRMATION OF THE PERIPLASMIC NATURE OF THE PROTEINS RELEASED BY PMBN

The results presented in this chapter so far suggest that PMBN exclusively releases periplasmic proteins from E.coli. This presumably results from outer membrane damage yet no evidence for release of outer membrane proteins has been obtained. The contention that no outer membrane proteins were released was examined by further separate experiments which aimed to show that the released proteins did not correspond to outer membrane

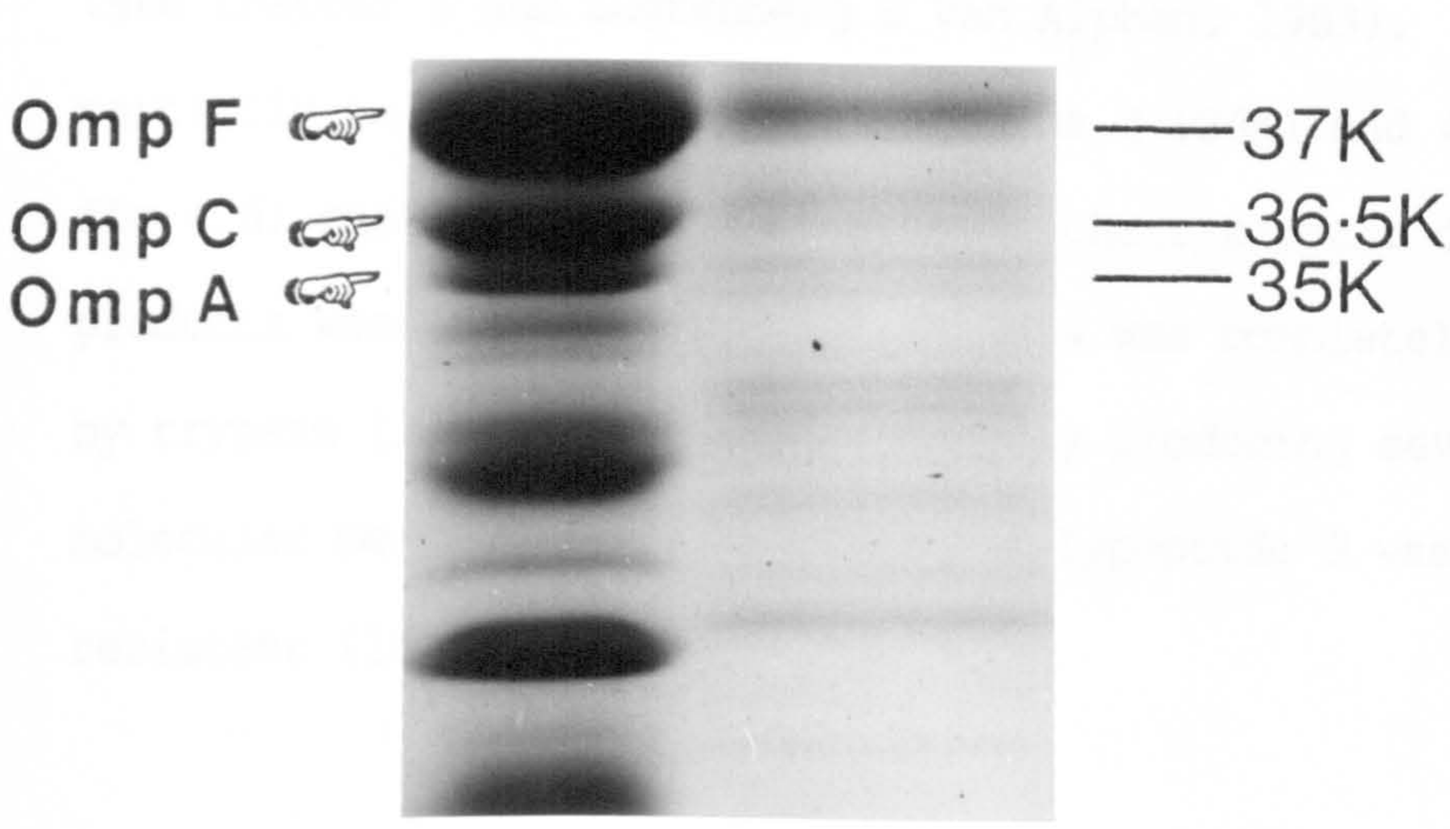


Fig 4.8 Comparison of polypeptides released by PMBN and the major outer membrane proteins of E.coli. The profile of outer membrane proteins prepared from E.coli K12 as described in the text is shown in lane a. The polypeptide profile of E.coli K12 following treatment with PMBN (200ug/ml) is shown in lane b. The major proteins associated with the outer membrane are shown on the left, molecular weights on the right.

proteins.

4.4.1 PMBN-released proteins - trypsin digestion:

The outer membrane protein OmpA (35K) in E.coli is digested by the proteolytic enzyme trypsin resulting in two low molecular weight fragments of 24K and 19K on SDS-PAGE (see Chapter 5 and Lugtenberg & Van Alphen, 1983). In order partially to characterise polypeptides 3 (36K) and 4 (lane e fig 4.6) that were released by PMBN, their tryptic digest products were examined. Polypeptide 4 was completely digested by trypsin (lane b fig 4.9) presumably producing several low molecular weight fragments whereas polypeptide 3 was trypsin resistant (lane b fig 4.9).

4.4.2 PMBN released proteins - heat modification:

Interactions between constituents of the outer membrane are very strong and require solubilisation temperatures above 70^0C for complete denaturation (Lugtenberg & Van Alphen, 1983). At temperatures below 70^0C, incomplete solubilisation leads to lowered electrophoretic mobility. Indeed, the apparent molecular weight of OmpA after boiling in 2% SDS is 35K, whereas the molecular weight of the non-denatured form is 28K (Lugtenberg & Van Alphen, 1983). The migration of the PMBN-released proteins after solubilisation at 60^0C or 100^0C was compared, but since no differences in their mobilities were observed (fig 4.10), clearly the proteins were not heat-modifiable. However, polypeptide 3 may be more efficiently solubilised at 100^0C because the

Fig 4.9 Cleavage of PMBN-released polypeptides by trypsin. PMBN (200ug/ml) released polypeptides 3 and 4 (lane a and see also fig 4.6). Cell suspensions of E.coli K12 3300 (pBR322) were incubated with trypsin (1mg/ml) for 20 min and the reaction terminated by the addition of soybean trypsin inhibitor (500ug/ml). The trypsin treated sample was lyophilized and subjected to electrophoresis (lane b). Proteins were detected by Coomassie blue. The figure shows that portion of the gel which contains periplasmic polypeptides 3 and 4. The position of these polypeptides in lane a is indicated by the arrows and numbers on the left.

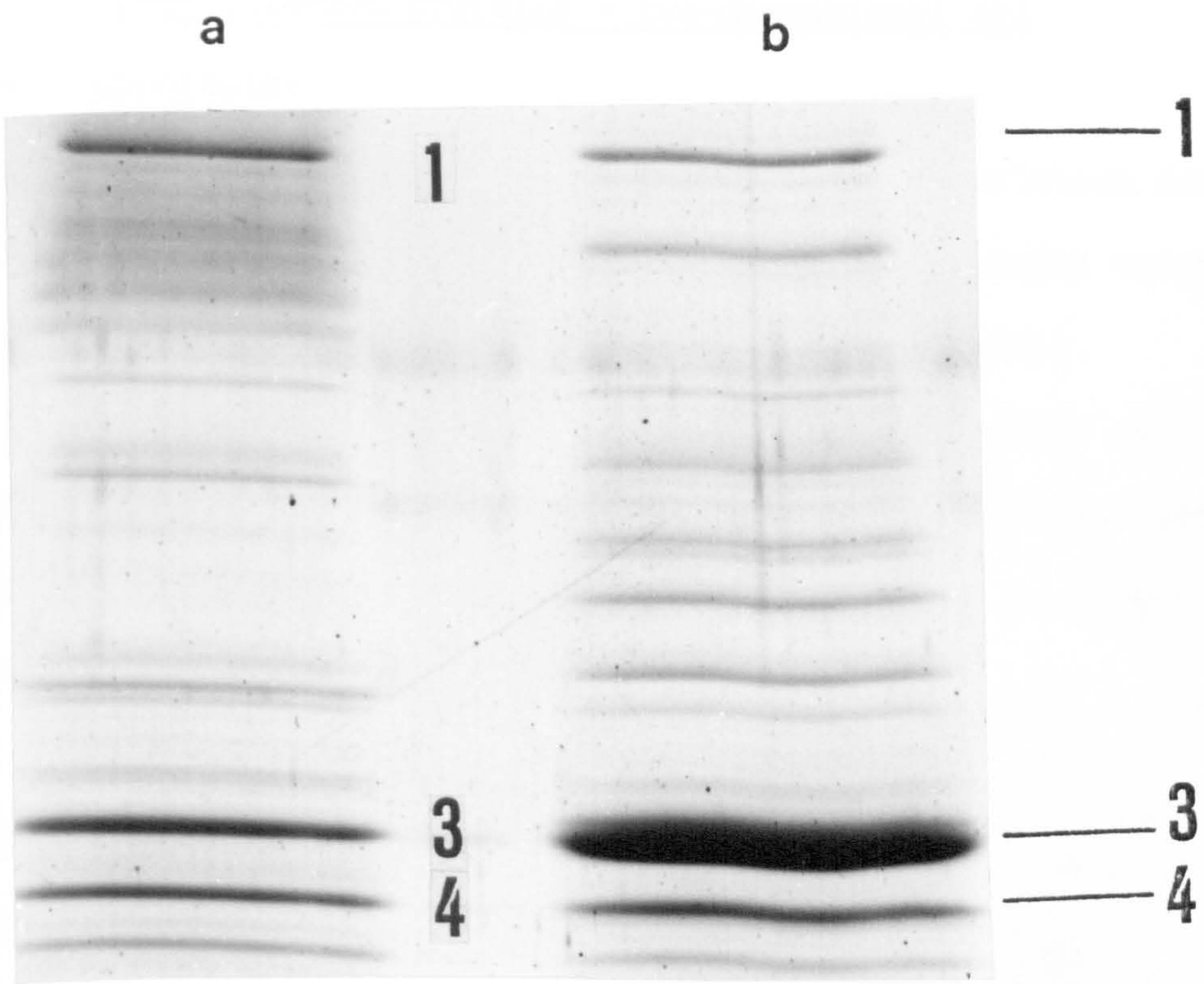


Fig 4.10 Heat modification of polypeptides released by PMBN. Polypeptides 3 and 4 (fig 4.6) were released from E.coli K12 3300 (pBR322) following treatment with PMBN. Lyophilised protein samples containing proteins 3 and 4 (arrowed) were solubilized at either 60^0C (lane a) or 100^0C (lane b)

intensity of this band was greater after denaturation of samples at the higher temperature (lane b fig 4.10).

4.4.3 PMBN-released proteins - two-dimensional gel electrophoresis:

Two-dimensional gel electrophoresis is a high resolution method for separation of proteins based on both the molecular weight and the isoelectric point of the protein (Hames 1981). Polypeptides 3 and 4 (lane e fig 4.6) released by PMBN were resolved as at least 5 separate spots on two-dimensional gels (data not shown). The pIs of these spots did not correspond either with OmpF (pI 5.0) or OmpC (pI-4.8).

4.4.4 PMBN-released proteins - characterisation by immunoblotting:

To examine further whether the proteins released from whole bacteria by PMBN might originate from the outer membrane, the proteins were subjected to immunoblotting using antisera directed against the major outer membrane proteins. Preparations obtained after treatment of bacteria with PMBN were electrophoresed on the same gel as outer membrane proteins prepared by the method of Yamato et al. (1975). The majority of polypeptides released by PMBN (lanes b and e fig 4.5) transferred easily to nitrocellulose paper by the method of Towbin et al. (1979). They were then probed with anti-OmpF/C serum (kindly provided by Dr. P. Owen, Trinity College, Dublin). The antiserum failed to detect major outer membrane proteins in the material released by PMBN (data not shown).

Unfortunately, however, the antiserum failed to detect OmpF or OmpC in control outer membrane preparations.

4.5 POSSIBLE RELEASE OF PROTEIN FROM ISOLATED MEMBRANE VESICLES AFTER POLYMYXIN B OR PMBN TREATMENT

The possibility that polymyxins might release major proteins from isolated outer or inner membrane preparations was examined. Outer and inner membranes were prepared by the method of Yamato et al., (1975) and treated with polymyxin B or PMBN for 60 min. Membranes were recovered by centrifugation and their polypeptide content examined and compared by electrophoresis with similar preparations which had not been treated with polymyxins. No evidence for release of membrane proteins by either compound was obtained (data not shown).

DISCUSSION

Several of the observations made in this chapter concerning the action of polymyxin B and PMBN are consistent with previously published findings. These include the ability of polymyxin B to release periplasmic and some cytoplasmic proteins (Cerny & Teuber, 1971 and 1972; Storm et al., 1977 and Vaara & Vaara, 1981) and the failure of both compounds to release LPS (Cerny & Teuber, 1972; Vaara & Vaara, 1981 and 1983b). However, in contrast to the previous studies of Vaara & Vaara (1983b) I have clearly shown that PMBN releases periplasmic proteins. Indeed the proteins released by PMBN are likely to be entirely derived from the periplasmic space because PMBN failed to cause leakage of the cytoplasmic marker enzyme beta-galactosidase and there was no evidence that it released either outer or inner membrane proteins from whole cells or isolated membrane vesicles.

As previously mentioned, Vaara & Vaara (1983b) also examined the question of whether PMBN releases periplasmic proteins. They followed the release of plasmid R471a-encoded beta-lactamase from Salmonella typhimurium, but PMBN even at concentrations as high as 100ug/ml apparently failed to release the enzyme (Vaara & Vaara, 1983b). However, the experimental system adopted in the present study to test for PMBN-induced beta-lactamase leakage is undoubtedly more sensitive than that employed by Vaara & Vaara. There are two principal reasons for the increased sensitivity of the system adopted in the present

study. Firstly, the use of pBR322 rather than R471a to encode beta-lactamase provides a higher level of periplasmic enzyme because the copy number of pBR322 (20 to 30 per cell) (Boyer et al., 1977) is considerably higher than that of R471a, which is a large self-transmissible plasmid likely to be present at only a few copies per cell (Hedges et al., 1975 and Bukhari et al., 1977). Secondly, the beta-lactamase assay adopted in the present study uses the chromogenic cephalosporin nitrocefin, whereas Vaara & Vaara used an iodometric assay for the enzyme (Vaara & Vaara, 1983b). The nitrocefin method is more sensitive than the iodometric system (O'Callaghan et al., 1972; Richmond & Sykes, 1973; Ross & O'Callaghan, 1975 and Plested, 1983).

Apart from the finding that PMBN releases periplasmic proteins from Gram-negative bacteria, another observation of interest concerns the difference in the ability of polymyxin B and PMBN to release a protein of molecular weight 36K from E.coli. Although no evidence for release of outer membrane proteins had been obtained, it was previously thought possible that polypeptide 3 released from E.coli following PMBN treatment might be one of the major outer membrane proteins, because its molecular weight (36K) was similar to that of the major outer membrane proteins OmpA, OmpF and OmpC (Lugtenberg & Van Alphen, 1983). More specifically it co-migrated with the OmpF protein. In support of the possibility that this protein is an outer membrane protein, it should be noted that osmotic shock procedures can, under certain circumstances, liberate proteins from other parts of the cell

(Jacobson et al., 1976). The migration of OmpA during electrophoresis in the presence of SDS varies with respect to the temperature of denaturation and the protein is also trypsin sensitive (Lugtenberg & Van Alphen, 1983). Polypeptide 3 (fig 4.6) released by PMBN was not digested by trypsin, nor was it heat modifiable. Although polypeptide 4 (fig 4.6) was trypsin-sensitive it was not heat modifiable. Furthermore the trypsin fragments of polypeptide 4 did not correspond to the fragments of molecular weights 19K or 24K reported for OmpA. These observations discount either polypeptide 3 or 4 as OmpA.

The 36K protein (polypeptide 3) released by PMBN was neither OmpF nor OmpC because its isoelectric point during two dimensional electrophoresis differed from OmpF (pI-5.0) and OmpC (pI-4.8) outer membrane proteins (Lugtenberg & Van Alphen, 1983). Two dimensional gel electrophoresis poses difficulties for the identification of OmpA, because the protein appears as at least twelve spots, most of which are caused by artefacts (Henning et al., 1978). Hence conclusive evidence that polypeptides 3 or 4 were or were not OmpA could not be obtained by this method. Transfer of the proteins released by PMBN onto nitrocellulose paper and probed with anti-serum purported to be specific for OmpF/C (Dr.P.Owen, personal communication) failed to provide conclusive evidence for the outer membrane origin of the PMBN-released proteins. The 36K protein preferentially released by PMBN is therefore very likely to be periplasmic although its identity as yet is unknown.

CHAPTER 5

FURTHER STUDIES OF THE EFFECTS OF POLYMYXIN B NONAPEPTIDE AND POLYMYXIN B ON THE CELL ENVELOPE

INTRODUCTION

In the previous chapter, the effects of polymyxin B and PMBN on cell envelope integrity were compared. Both compounds caused loss of proteins from E.coli K12 3300 (pBR322) although PMBN released less protein than did polymyxin B. The individual proteins released by either compound were different but were derived principally from the periplasm accompanied in the case of polymyxin B by a low level of cytoplasmic proteins (see Chapter 4). The leakage of periplasmic proteins induced by PMBN indicates that it probably causes disruption of the outer membrane which results in modification of the barrier functions of the envelope. These modifications, following treatment with agents known to disturb the structure of the outer membrane, are well documented (see Hancock, 1984 and Nikaido & Vaara, 1985 for reviews). Treatment of Gram-negative bacteria with, for example, an appropriate concentration of EDTA increases the permeability of the cells to a wide range of antimicrobials, lysozyme and other enzymes but nevertheless has no effect on viability (see review of Wilkinson, 1975).

This chapter describes experiments which show that PMBN -treated cells are more permeable to certain compounds that are normally excluded by the intact outer membrane of the cell. To date, studies on the permeability changes mediated by PMBN involving increased influx have been restricted to low molecular weight hydrophobic antibiotics (see Chapter 3; Vaara & Vaara, 1983a and c; Nikaido & Vaara, 1985 and Vaara et al., 1985). The experiments described here extend the findings of

these workers and provide information on the effects of PMBN treatment on the subsequent uptake of the hydrophobic dye crystal violet and the enzyme lysozyme.

The potential ability of PMBN to alter the orientation or topographical arrangment of membrane proteins has also been studied, as well as the possibility that PMBN interaction with membranes might lead to denaturation of membrane enzymes. E coli K12 contains the outer membrane protein OmpA which is partially exposed at the surface of the cell. OmpA is important for maintenance of the structural integrity of the outer membrane, but unlike the other major proteins is susceptible to the proteolytic enzyme trypsin (Hammond et al., 1984). N-terminal parts of the protein however, appear to be embedded in the outer membrane since they remain intact following trypsin treatment in vivo, whereas isolated OmpA is completely digested. Thus, the trypsin cleavage site when OmpA is membrane bound is located outside the cell (fig 5.1). The complete amino acid sequence of the OmpA protein has now been determined (Chen et al., 1980) and the domains associated with the outer membrane have been identified. The ability of PMBN to alter the accessibility of OmpA protein to trypsin has been considered in this chapter. Beta-lactam antibiotics bind covalently to membrane located target enzymes, the penicillin binding proteins (PBPs), an association that leads ultimately to bacterial death (Gale et al., 1981). The PBPs contribute to the normal enzymic activities necessary to maintain cell integrity, growth and shape. In E.coli, benzyl penicillin binds to at least seven different PBPs located in the

Fig 5.1 Hypothetical structure of the Gram-negative cell envelope. The possible disruption caused by the intercalation of PMBN and its effects on the accessibility of OmpA to trypsin (see text).

Abbreviations:-

a = OmpA with trypsin cleavage site.

t = trypsin.

lps = lipopolysaccharide.

pl = phospholipid.

p = periplasmic protein.

pg = peptidoglycan.

bl = bound lipoprotein.

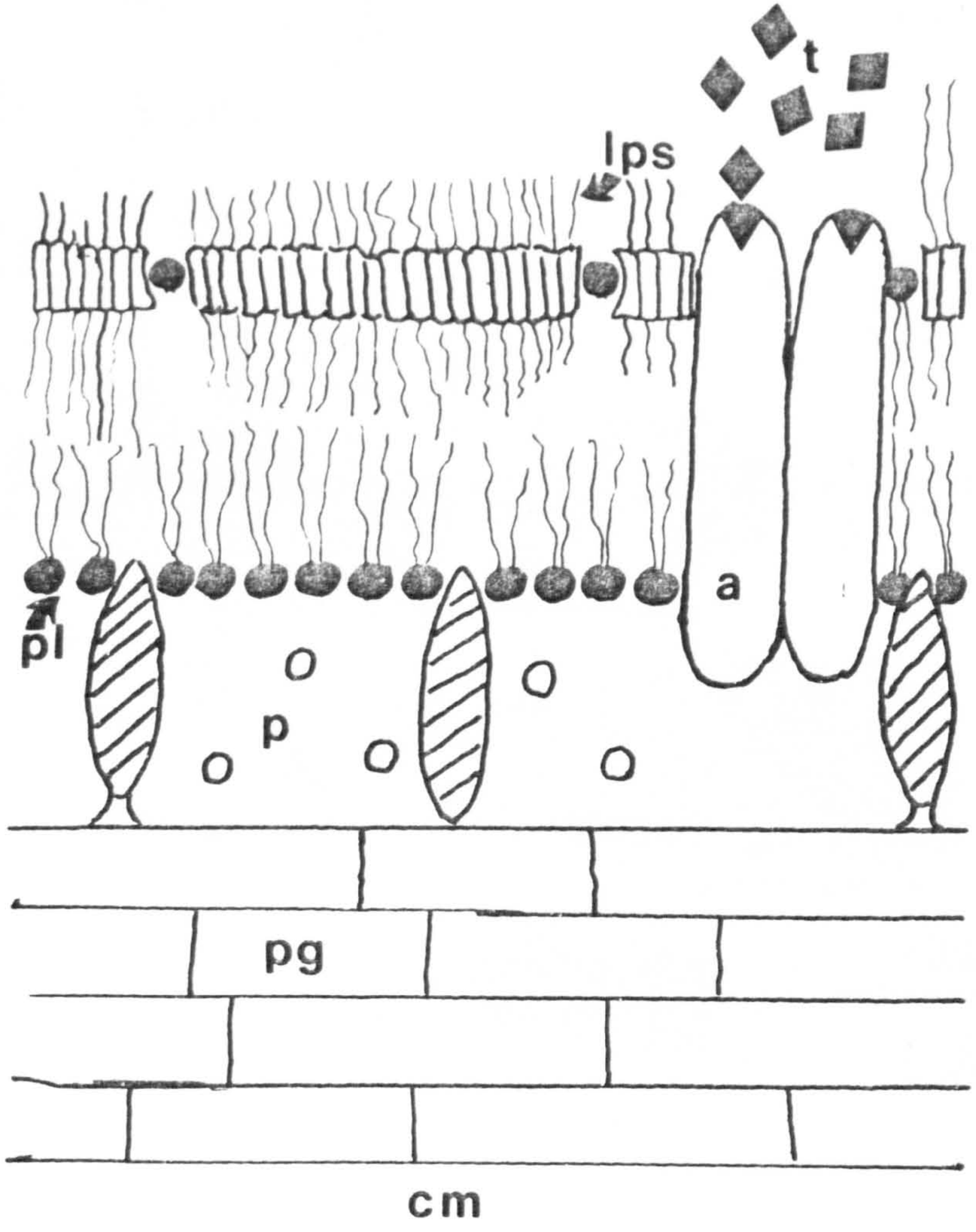
fl = free lipoprotein.

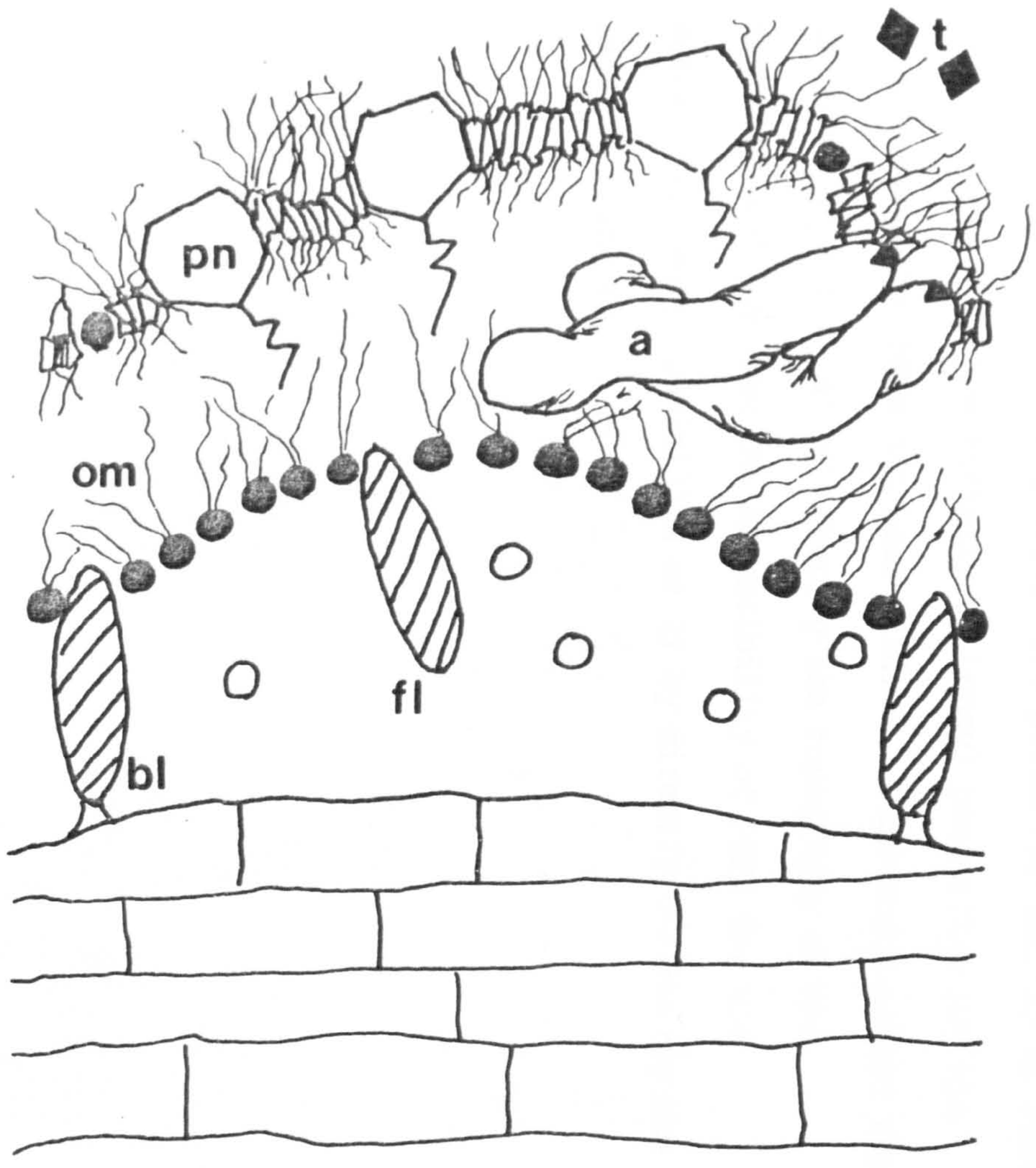
om = outer membrane.

cm = cytoplasmic membrane.

pn = PMBN.

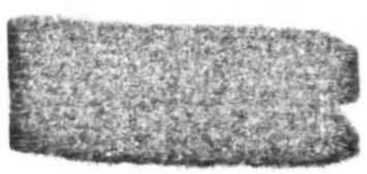
● = divalent cations.


lps
t
a
pl
p
pg
cm


t
pn
a
om
fl
bl

cytoplasmic membrane (fig 5.2). The potential ability of PMBN to interfere with the affinity of benzyl penicillin for the membrane-located PBPs has been studied. PMBN might affect this interaction either a) by altering the topography of the membrane and changing the accessibility of PBPs for the substrate, benzyl penicillin or 2) by directly denaturing the enzymes involved.

Fig 5.2 Properties of the penicillin binding proteins (PBPs) of E.coli K12 (data from Spratt 1977).

	PBP	Molecular Weight	Molecules per cell	Gene and map position (min)		Cellular function
	1a	91,000		ponA	73·5	PBPs 1a/1bs are 'essential' proteins involved in the biosynthesis of peptidoglycan associated with cell elongation. Combined inhibition causes spheroplasting and lysis.
	1bs	86,500 84,000 81,500	230	ponB	3·3	
	2	66,000	20	rodA	14·4	'Essential' protein involved in shape maintainance. Inhibition leads to 'round-form' production and eventually cell death.
	3	60,000	50	fts	1·8	'Essential' protein involved in cell division. Inhibition leads to filamentation and eventually cell death.
	4	49,000	110	dacB	68	'Non-essential'. Responsible for D-alanine carboxypeptidase 1B-C activity.
	5	42,000	1 800	dacA	13·7	'Non-essential'. Responsible for D-alanine carboxypeptidase 1A activity.
	6	40,000	570	?	?	'Non-essential' (?). Not involved in β-lactam action.

RESULTS

5.1 PMBN ENHANCES CELL ENVELOPE PERMEABILITY TO LYSOZYME

Lysozyme is a protein of molecular weight 14K which acts antibacterially by cleaving the sugar backbone of peptidoglycan in the bacterial cell wall. It is effective against Gram-positive organisms but fails to inhibit Gram- negative bacteria because lysozyme cannot normally penetrate their outer membranes. Exposure to EDTA destabilises the outer membrane sufficiently to allow lysozyme to enter and after peptidoglycan degradation cell lysis occurs (DiRienzo et al., 1978 and Leive, 1974). To establish whether PMBN also has this destabilising property, a direct comparison of the effects of EDTA and PMBN on lysozyme-mediated cell lysis was undertaken.

Concentrated suspensions of E.coli K12 3300 (pBR322) (10^{10} bacteria per ml) were exposed to freshly prepared solutions of lysozyme (0.4ug/ml). Either PMBN (200ug/ml) or EDTA (0.3mM final concentration) was then added, the cells diluted and absorbance at a wavelength of 675nm determined at frequent intervals following addition of the compounds. Addition of EDTA caused lysis of the lysozyme-treated cells which was reflected by a rapid and sustained fall in absorbance values over 50 min (fig 5.3). PMBN treatment also resulted in lysis of the lysozyme-treated cells over the time period, although the effect was less pronounced than with EDTA (fig 5.3). Clearly, in comparison with control cells, treatment with either EDTA or PMBN caused lysis of the lysozyme-treated

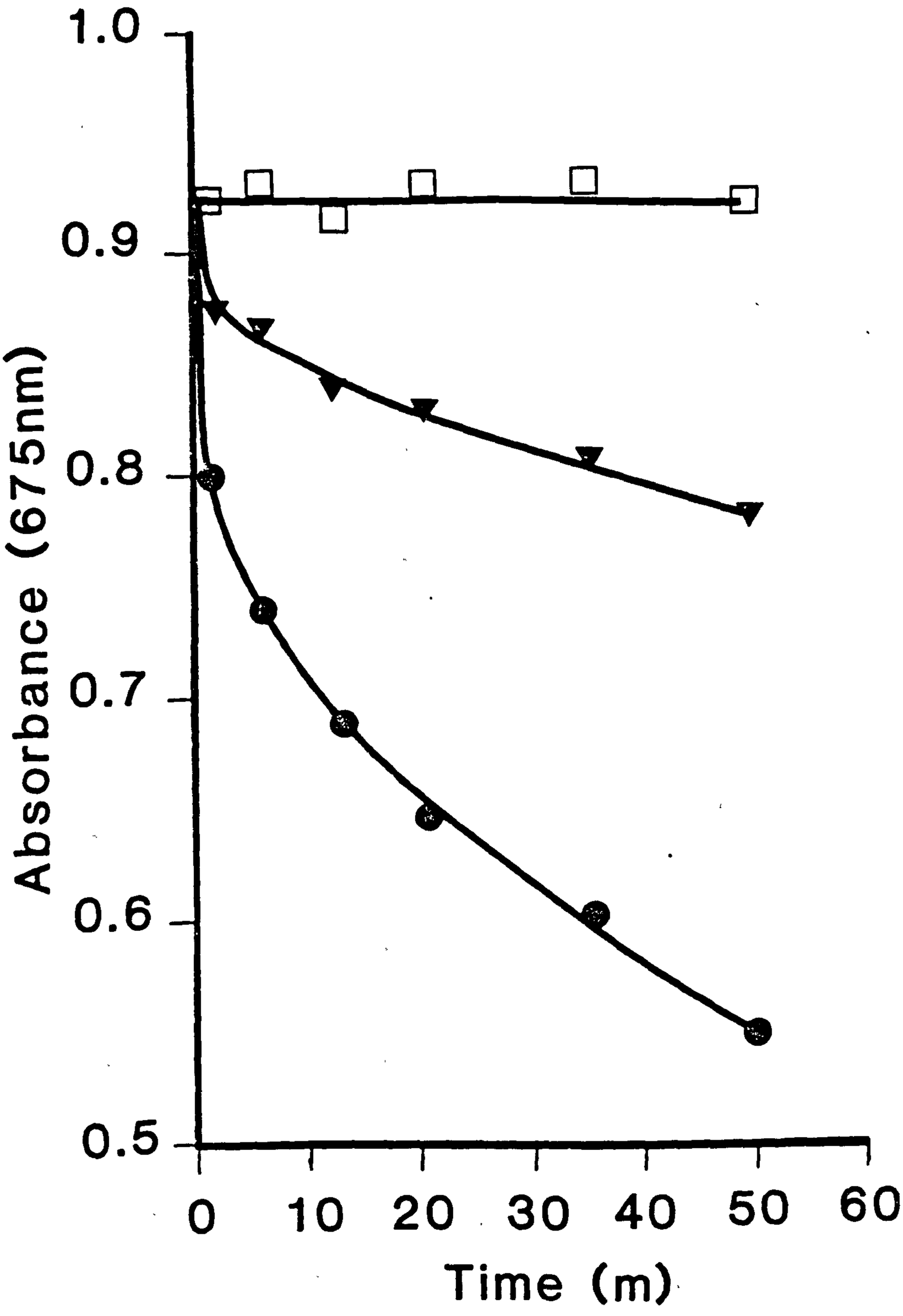


Fig 5.3 The effects of PMBN on lysozyme-treated E.coli K12 3300 (pBR322). Absorbance values of cell suspensions previously exposed to lysozyme (□) control cells. Treatment at time zero with EDTA (0.3mM) (●) or PMBN (200ug/ml) (▼).

cells, (fig 5.3) thus confirming that PMBN promotes lysozyme entry to the peptidoglycan.

5.2 PMBN ENHANCES ENVELOPE PERMEABILITY TO THE HYDROPHOBIC DYE-CRYSTAL VIOLET

The hydrophobic dye crystal violet (molecular weight 408) cannot normally penetrate the outer membrane of Gram-negative cells (Gustafsson et al., 1973 and Hancock, 1984). However, pre-treatment with agents capable of disturbing the barrier function of the outer membrane permits the uptake of crystal (or gentian) violet (Newton, 1954; Gustafsson et al., 1973; Vaara & Vaara, 1981 and Hancock, 1984).

The uptake of crystal violet was followed in E.coli K12 3300 (pBR322). Organisms at the mid-logarithmic stage of growth were incubated with 10ug/ml of crystal violet at 37^0C. for exactly 30 min. The dye remaining in the cell-free supernatant was assayed spectrophotometrically at 590nm according to the method of Gustafsson et al., 1973). Untreated control cells bound little crystal violet with the result that the majority of the dye remained in the supernatant. However, the uptake of crystal violet by bacteria treated with either EDTA or polymyxin B was increased, resulting in a lower concentration of dye in the cell-free supernatant. Pre-treatment with polymyxin B (200ug/ml) induced a 58% uptake of the added dye, whilst EDTA (1mM) treatment resulted in only a 31% uptake (table 5.1). Pre-treatment of the cells with PMBN also caused an increase in the uptake of

TABLE 5.1

THE EFFECTS OF PMBN AND POLYMYXIN B ON THE PERMEABILITY OF E.COLI K12 3300 (pBR322) TO CRYSTAL VIOLET

	TIME (m)	ABSORBANCE (590nm)	UPTAKE (%)
CONTROL UNTREATED	0	1.66	--
CONTROL UNTREATED	30	1.38	17
POLYMYXIN B (200ug/ml)	30	0.69	58
EDTA (1mM)	30	1.14	31
PMBN (200ug/ml)	30	0.62	63

Organisms at the mid-logarithmic stage of growth were incubated at 37^0C with 10ug/ml of crystal violet for exactly 30 min. The dye remaining in the cell-free supernatant was assayed spectrophotometrically.

crystal violet by E.coli. After 30 min at 37^0C. 63% of the dye was taken up by the PMBN-treated cells, compared with only 17% uptake by the untreated control (table 5.1).

5.3 THE EFFECTS OF PMBN ON TRYPSIN DIGESTION OF OUTER MEMBRANE PROTEINS

The proteolytic enzyme trypsin although partially excluded from the cell due to its size nevertheless has the ability to release certain outer membrane proteins. To establish whether PMBN interferes with trypsin-promoted cleavage of major outer membrane proteins in whole cells, bacteria were exposed to trypsin, following PMBN treatment and membrane fractions from the cell isolated.

E.coli K12 3300 (pBR322) (10^{10} bacteria/ml) was incubated with trypsin (1mg/ml) following pre-treatment with 200ug/ml of PMBN for 1hr at 37^0C. Envelope fractions were then prepared from these cells by conventional procedures (see Chapter 2). Outer membrane proteins from samples were separated on SDS-PAGE and the gels scanned to produce densitometer traces (fig 5.4). The electrophoretic mobilities of OmpF/C and OmpA (fig 5.4A) were consistent with previously published work (Chopra & Eccles, 1978). In contrast, polypeptide profiles from the outer membrane of cells which had been treated with trypsin (fig 5.4B) clearly showed the absence of OmpA and the appearance of its low molecular weight degradation product which has been described previously (Lugtenberg & Van Alphen, 1983). However, treatment of

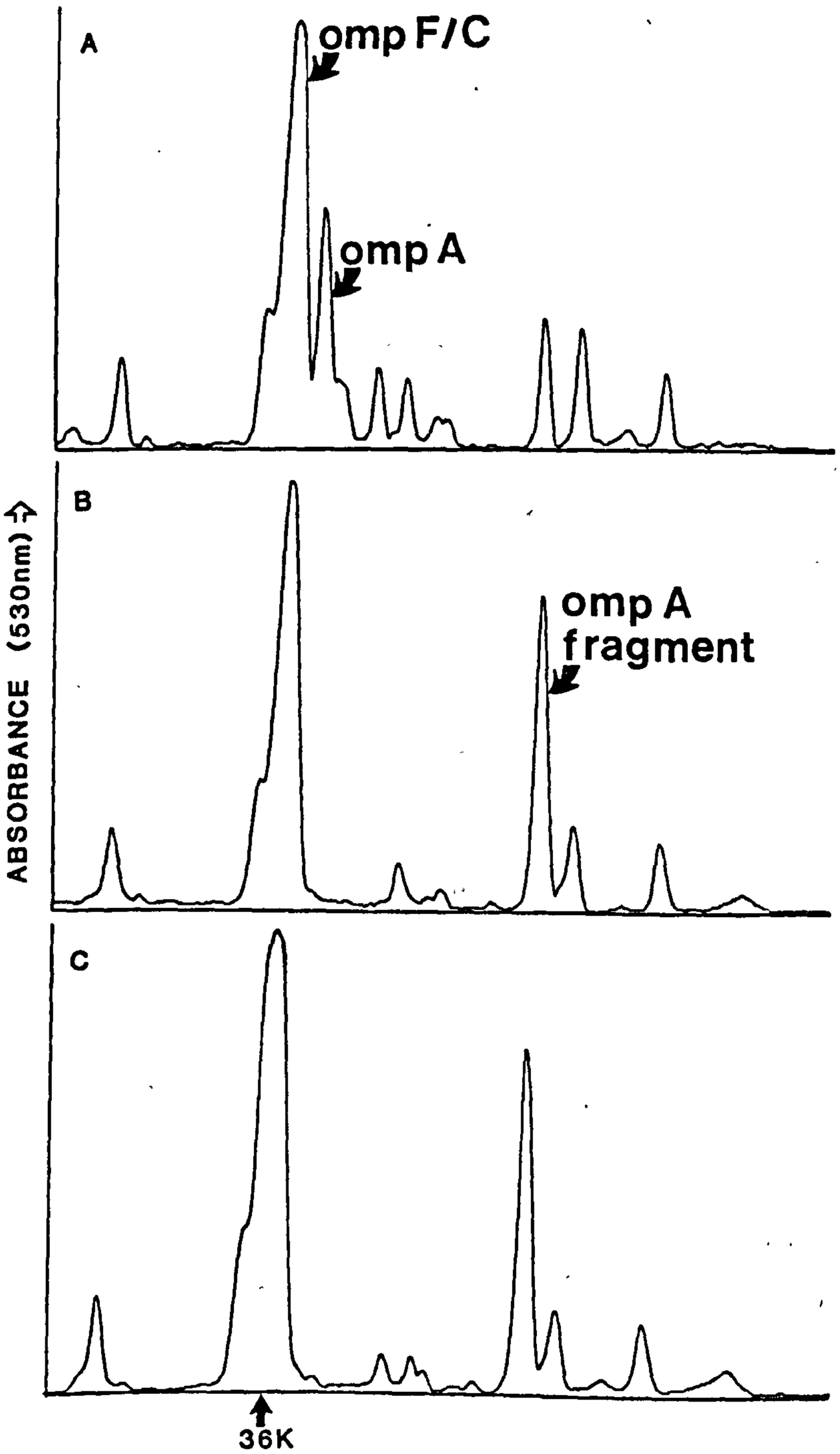


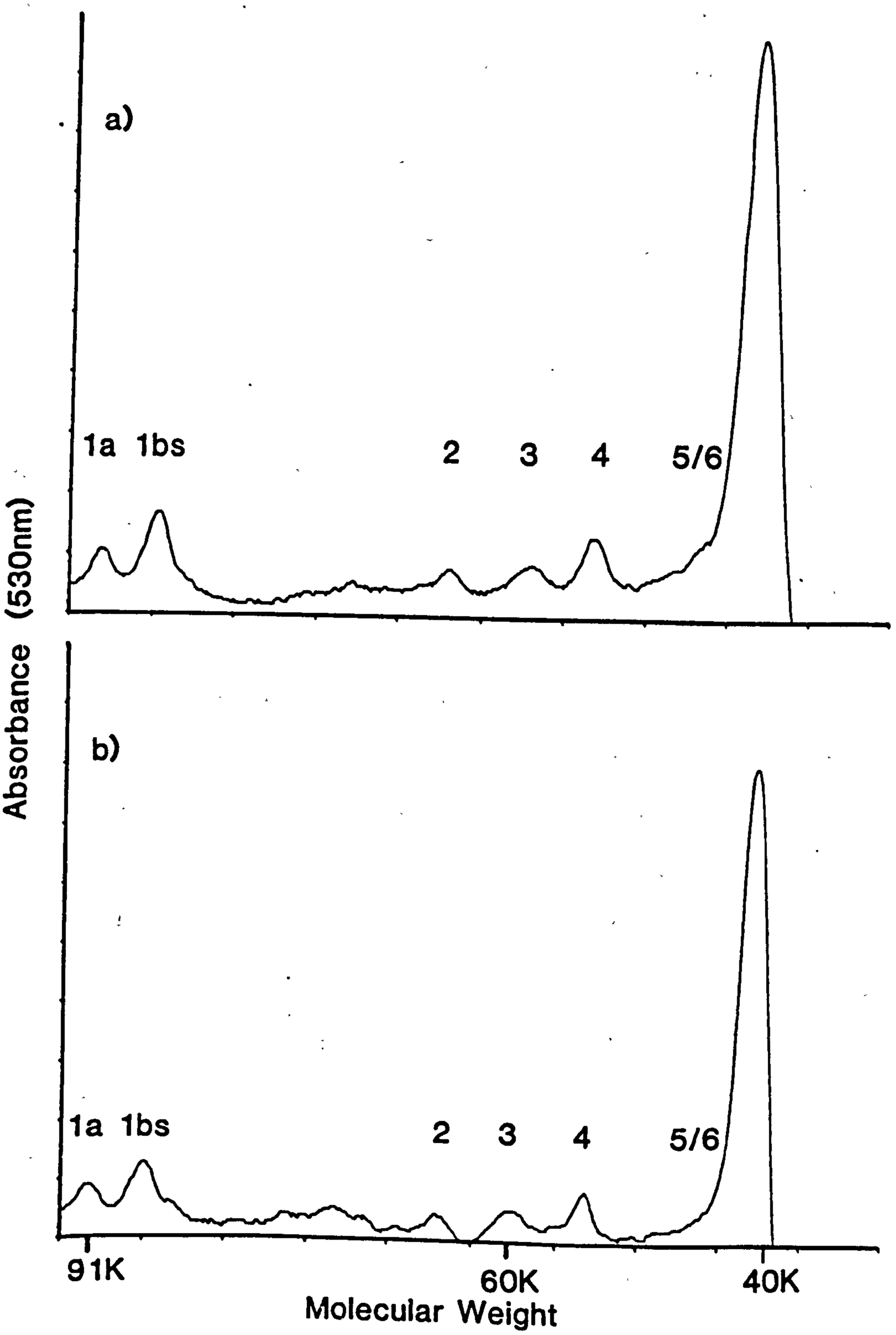
Fig 5.4 The effects of PMBN on trypsin digestion of outer membrane proteins. Densitometer scans of outer membrane proteins from isolated envelopes (**A**); treated with trypsin alone (**B**) or PMBN (200ug/ml) and trypsin (**C**). For details of experiment see text.

bacteria with PMBN did not alter the ability of trypsin to degrade OmpA protein (fig 5.4C).

5.4 EFFECTS OF PMBN ON AFFINITY OF BENZYL PENICILLIN FOR PENICILLIN BINDING PROTEINS (PBPs) IN ISOLATED MEMBRANES

Benzyl penicillin binds to penicillin binding proteins (PBPs) located in the cytoplasmic membrane of all penicillin-susceptible bacteria. The PBPs represent the targets of beta-lactam-induced killing of bacteria (Gale et al., 1981). To establish whether PMBN interferes with the affinity of penicillin for these proteins, envelope fractions from E.coli K12 3300 were isolated and treated in vitro with 200ug/ml of PMBN. Proteins from samples which had been treated with PMBN were exposed to ^{14}C-labelled benzyl penicillin and subjected to SDS-PAGE followed by autoradiography (see Chapter 2 for details). Densitometer scans of the negatives (fig 5.5) showed at least 7 major peaks representing those PBPs previously identified in E.coli K12 strains (Spratt, 1977). However, some degradation during the course of the experiments appeared to have produced additional minor peaks in both scans. Nevertheless there were no qualitative or quantitative differences between PMBN-treated and untreated membrane preparations.

Fig 5.5 Densitometer scans of the penicillin binding proteins (PBPs) of E.coli. Envelope fractions from E.coli K12 3300 were isolated and treated in vitro with 200ug/ml of PMBN as described in Chapter 2. Peaks (1a - 6) representing PBPs following treatment with PMBN are shown in (a). Untreated control PBPs are shown in (b).


a)
1a
1bs
2
3
4
5/6
(530nm)
Absorbance
b)
1a
1bs
2
3
4
5/6
91K
60K
40K
Molecular Weight

DISCUSSION

Results presented here indicate that both PMBN and EDTA increase the permeability of the E.coli outer membrane to lysozyme (fig 5.3). The outer membrane disrupting action of PMBN appears to be similar to polymyxin B in this respect. The bactericidal agent polymyxin B allows access of lysozyme (Teuber, 1970 and Vaara & Vaara, 1981), although the membrane disorganising effects of this agent are generally masked by its direct lethal action (Vaara & Vaara, 1983a).

There are various chemical treatments which can alter the penetration barrier of Gram-negative organisms sufficiently to allow them to become susceptible to a range of hydrophobic agents to which they were previously insensitive. For example, in EDTA-treated cells the barrier function of the outer membrane becomes impaired. Leive et al., (1968 and 1974) reported that treatment with low levels of Tris-EDTA resulted in cells which released up to 50% of their LPS and some protein. These cells retained viability but became vulnerable to hydrophobic agents such as actinomycin D, novobiocin and rifampicin to which they were not previously susceptible. The stability of the outer membrane appears to depend upon the presence of divalent ions which link adjacent LPS molecules. Removal of these ions by EDTA chelation results in membrane disruption. The ensuing loss of LPS apparently leads to the breakdown of the penetration barrier and results in an increase in the permeability of the cell to hydrophobic agents and detergents. When cells were exposed to lysozyme, EDTA (0.3mM)

induced a similar degree of lysis as PMBN (fig 5.3), although the mode of action of the two agents appears to be different. Unlike EDTA, the outer membrane disrupting action of PMBN (or polymyxin B) is not associated with the release of LPS from E.coli (see Chapter 4 and Vaara & Vaara, 1983b).

Normally the basic hydrophobic dye crystal violet does not easily penetrate the intact outer membrane, but is able to do so without difficulty when the bacteria are damaged by agents such as EDTA or polymyxin B (Vaara & Vaara, 1981). Treatment of E.coli with either of these agents or PMBN resulted in enhanced permeability of the cells to crystal violet (table 5.1). EDTA at a concentration of 1mM promoted crystal violet uptake (31%), whereas values for the uptake by untreated (control) cells (17%) were consistent with reports by Gustafsson et al., (1973) and Vaara & Vaara, (1981), binding being ascribed to adsorption (but not penetration) of the dye to the bacterial cell surface. Treatment of cells with polymyxin B resulted in crystal violet uptake (50%; table 5.1) consistent with the findings of others (Vaara & Vaara, 1981) that polymyxin B disrupts the outer membrane to allow access of hydrophobic agents. PMBN when tested at a concentration (200ug/ml) equivalent to polymyxin B promoted slightly more uptake (63%). The extent of outer membrane disturbance caused by PMBN may well account for the rapid sensitisation of PMBN-treated cells to hydrophobic antibiotics (see Chapter 3 and Vaara & Vaara, 1983b).

The potential ability of PMBN to alter the orientation of a major outer membrane protein was investigated. It would seem

plausible that perturbation of the cell envelope by the intercalation of PMBN might change the orientation of OmpA protein. This could allow the exposed cleavage site to become inaccessible to trypsin (fig 5.1) or alternatively expose digestion sites previously buried in the outer membrane bilayer. The OmpA protein of E.coli has been well studied (see introduction to this chapter) and its cleavage by trypsin established. However, results presented in this chapter demonstrated (fig 5.4) that treatment of bacteria with PMBN did not alter the pattern of OmpA trypsin degradation products.

The ability of PMBN to interfere with the affinity of benzyl penicillin for PBPs was also investigated. The results (fig 5.5) demonstrated that PMBN did not affect the accessibility of PBPs for benzyl penicillin. In addition, PMBN did not denature the binding proteins.

CHAPTER 6

ALTERATION OF CYTOPLASMIC MEMBRANE PERMEABILITY INDUCED BY POLYMYXIN B NONAPEPTIDE AND POLYMYXIN B

INTRODUCTION

The previous chapter compared the effects of PMBN, polymyxin B and EDTA on the permeability of E.coli to various probes. Increased permeability of the cells to the dye crystal violet and lysozyme, to which they were normally impermeable, resulted from treatment with the polymyxins or EDTA. Outer membrane perturbation appeared to be responsible for this increase in permeability, although the exact mechanism(s) of such modification is not known at the present time.

Polymyxin B disrupts both outer and inner membranes in Gram-negative cells (see previous chapters; Newton, 1956; Cerny & Teuber, 1971, 1972; Storm et al., 1977; Klemperer et al., 1979 and Gale et al., 1981) and its mode of action is undoubtedly related to its membrane damaging effects (Storm et al., 1977 and Gale et al., 1981). However, whether damage to the outer membrane is more critical for cell killing than damage to the cytoplasmic (inner) membrane has not yet been clearly established.

Outer membrane disruption resulting from polymyxin B treatment is similar to that produced by its deacylated derivative PMBN, i.e. both compounds cause leakage of periplasmic proteins. Since PMBN has very low intrinsic antibacterial activity (see Chapter 3 and Nikaido and Vaara, 1985) outer membrane damage is unlikely to be directly responsible for the bactericidal action of polymyxin B. Therefore, the lethality of polymyxin B for Gram-negative organisms appears to be the consequence of cytoplasmic membrane

disruption. To test this hypothesis further, experiments were performed to compare the ability of polymyxin B and PMBN to damage the cytoplasmic membrane of E.coli. This chapter describes these experiments. The work was approached from the following viewpoint: If the lethal effect of polymyxin B arises from cytoplasmic membrane damage then it should cause significant changes in the permeability of that membrane, whereas the non-lethal compound PMBN is predicted to have no major effect on it.

As a general approach, changes in cytoplasmic membrane permeability resulting from damage by membrane-active agents can be studied by detecting the leakage of small molecules from the cell. These molecules, for example amino acids, are normally retained in their free state by washed suspensions of cells and have been released from this 'pool' in response to treatment by, for example, the polymyxins (Newton, 1956) or chlorhexidine (Hugo, 1967). In the present study, the effects of polymyxin B and PMBN on the retention of 'pools' were studied. In addition, another effect that reflects changes in membrane integrity is efflux of potassium ion (K^+) from cells. This leakage can be monitored by the use of a specific potassium ion electrode (Lannigan & Bryan, 1985). The release of K^+ from polymyxin B and PMBN-treated cells was examined. Furthermore, in some of the experiments to be described in this chapter the bactericidal agent chlorhexidine was used for reference (control) purposes, since it is known to produce cytoplasmic membrane damage (Hugo, 1982 and Lannigan & Bryan, 1985).

RESULTS

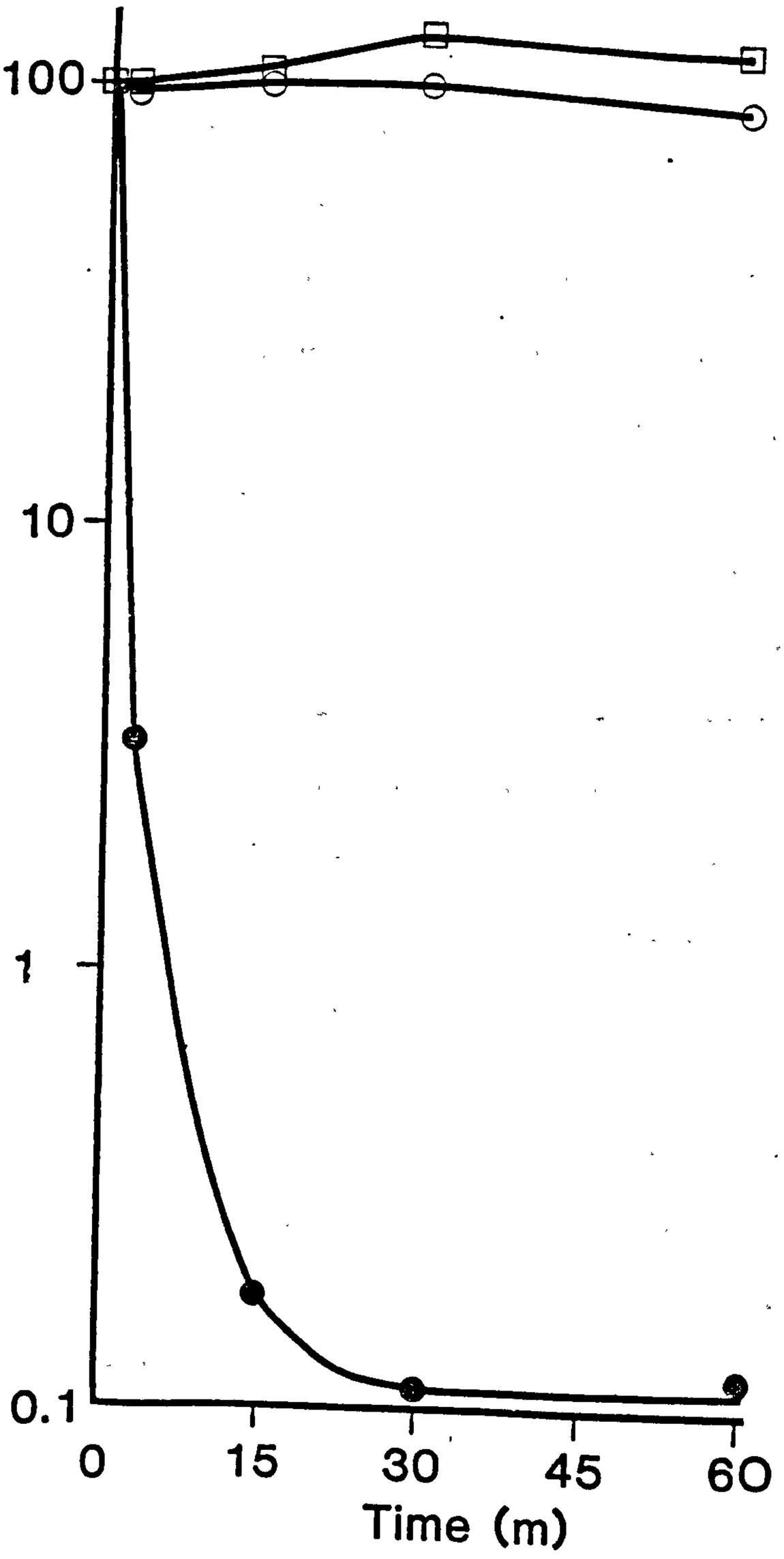
6.1 EFFECTS OF POLYMYXIN B AND PMBN ON GROWTH AND VIABILITY OF E.COLI

PMBN had a very low intrinsic antibacterial activity and was at least 300-fold less active than polymyxin B (see Chapter 3). Viability determinations following exposure to polymyxin B or PMBN were performed on E.coli K12 3300 (pBR322) suspended at 10^{10} cells per ml in 10mM sodium phosphate buffer (pH 7.4) These conditions are identical to those employed for the studies on cytoplasmic membrane damage (see below) and also relate exactly to the previous work on protein release from this strain, mediated by polymyxin B and PMBN (see Chapter 4). As already noted exposure to polymyxin B (200ug/ml) led to rapid loss of E.coli viability but an equivalent concentration of PMBN had no killing effect (see fig 3.7 which is also reproduced here as fig 6.1)

6.2 LOSS OF AMINO ACIDS, URACIL AND K^+ FROM POLYMYXIN B AND PMBN-TREATED BACTERIA.

As already noted in the introduction to this chapter, it is well established that bacterial cytoplasmic membrane damage can be monitored by determining the loss of small (soluble) molecules and/or K^+ from cells.

Fig 6.1 Viability of E.coli K12 3300 (pBR322) after exposure to polymyxin B or PMBN. Bacteria were grown to the mid logarithmic phase, harvested by centrifugation, washed in 10mM sodium phosphate buffer (pH 7.4) and then suspended in the same buffer to a density of 10^{10}/ml. The cell suspension was divided into three samples. At time zero, polymyxin B (200ug/ml) was added to one portion (●), and PMBN (200ug/ml) to the second (O). The third sample served as an untreated control (□). Bacteria were incubated at 37^{0}C and samples removed at the times indicated. Samples were rapidly diluted and then plated onto nutrient agar to determine viable bacteria. Values are the means of replicate determinations.


100
10
1
0.1
% Viable Bacteria
0
15
30
45
60
Time (m)

6.2.1 Leakage of amino acids

A mixture of ^{3}H-labelled amino acids was used to produce a labelled amino acid pool within E.coli K12 3300 (pBR322). Loss of amino acids from this pool was then followed by standard procedures (Chapter 2 and Britten & McClure, 1962). Approximate rates of radioactive loss were determined from the curves shown in fig 6.2. Untreated bacteria showed a low level of amino acid loss which exhibited biphasic kinetics (fig 6.2). During the first phase (lasting approximately 20 min) amino acid loss was relatively rapid (28 c.p.m. radioactivity lost/min), but this was followed by a second phase in which the rate of loss was very much slower (2 c.p.m. lost/min) (fig 6.2). Compared to the controls, polymyxin B (200ug/ml) and PMBN (200ug/ml) treatments resulted in enhanced rates of amino acid loss from E.coli (fig 6.2). This was reflected by accelerated loss in both the primary and secondary phases of amino acid leakage. In the primary phase the rate of PMBN-promoted loss was 65 c.p.m./min and in the secondary phase 11 c.p.m./min. The rate of amino acid loss promoted by polymyxin B in the primary phase (153 c.p.m./min) was approximately two-fold greater than that caused by PMBN (65 c.p.m./min). However the rates of secondary phase loss promoted by both compounds were similar (about 11 c.p.m./min in each case), but greater than the rate of loss from untreated (control) bacteria (2 c.p.m./min).

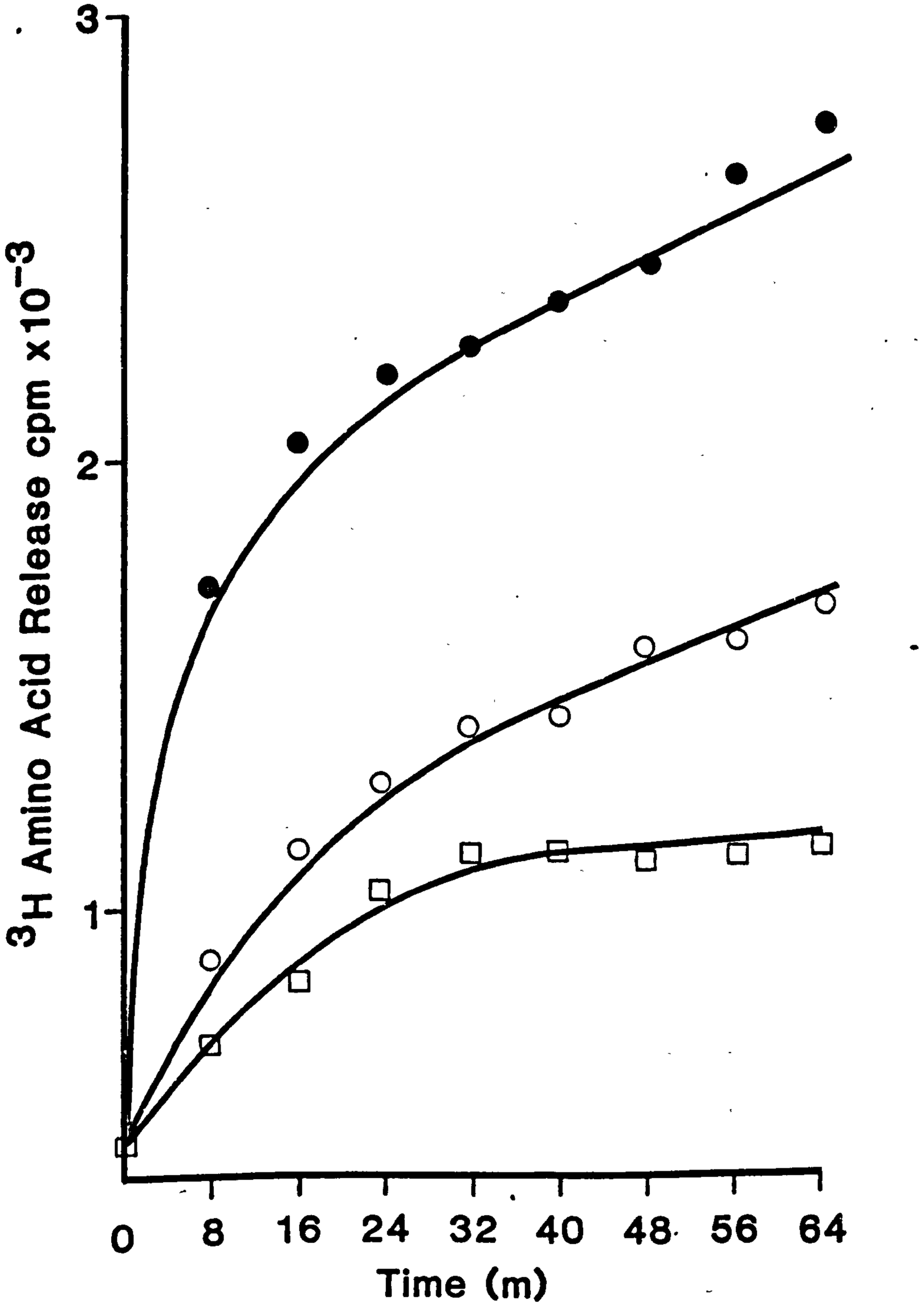


Fig 6.2 Release of free (pool) amino acids from E.coli K12 3300 (pBR322). Bacteria were grown to mid logarithmic phase and then incubated for 60 min with chloramphenicol 100ug/ml and ^{3}-H amino acids (2uCi/ml) to form a labelled amino acid pool. Bacteria were then prepared and exposed to polymyxin B (200ug/ml) (●) or PMBN 200ug/ml (O) as described in fig 6.1. A third sample served as an untreated control (□). Samples were removed at the time indicated and processed as described in the text.

6.2.2 Leakage of uracil

E.coli K12 3300 (pBR322) was also labelled with 6-^{3}H-uracil to follow loss of uracil from the cytoplasmic pool (Dougherty & Saukkonen, 1985). The results (fig 6.3) were comparable with those obtained for amino acid loss. Control (untreated) bacteria showed a low level of loss which displayed biphasic kinetics. Polymyxin B (200ug/ml) and PMBN (200ug/ml) both considerably increased the rates of loss of uracil from E.coli (fig 6.3). The rate of loss in the primary phase promoted by polymyxin B was approximately 1.5-fold greater than that caused by PMBN (fig 6.3)

6.2.3 Potassium ion loss

K^+ loss was investigated under conditions comparable to those used for amino acid and uracil leakage. Polymyxin B (200ug/ml) caused an immediate loss of K^+ (fig 6.4) which was complete within the first minute of exposure to the antibiotic (fig 6.4). PMBN (200ug/ml) also caused immediate K^+ leakage (fig 6.4). However, the loss of K^+ promoted by polymyxin B was approximately 2-fold greater than that caused by PMBN. Control (untreated) bacteria showed no K^+ loss (fig 6.4).

E.coli cells prepared in 10mM sodium phosphate buffer (pH 7.4) as detailed above were treated with a concentration of chlorhexidine (200ug/ml) equivalent to that already used for polymyxin B. Chlorhexidine at 200ug/ml induced an immediate leakage of K^+ although complete release of K^+ was delayed until 4 min after exposure to the agent (fig 6.5).

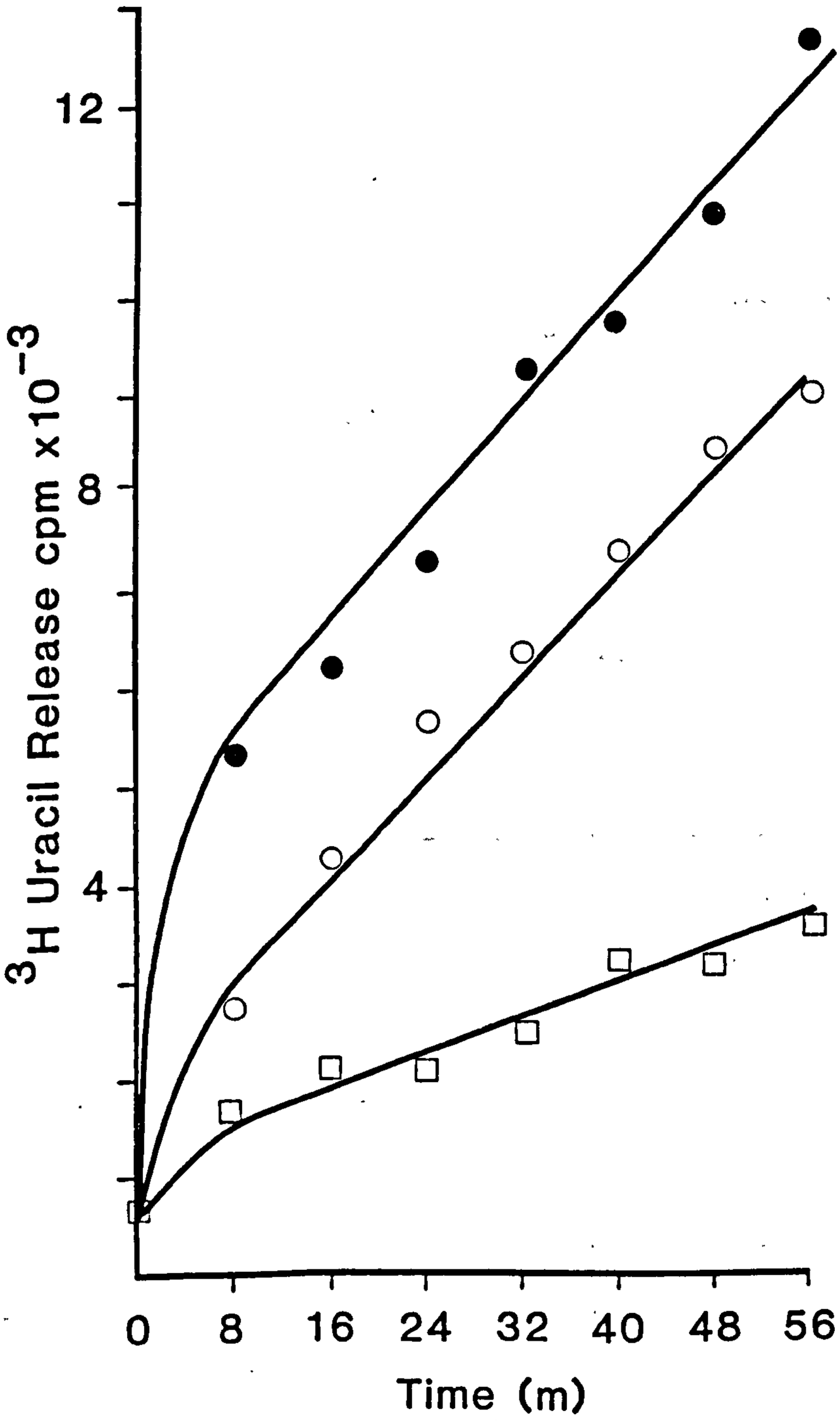


Fig 6.3 Release of free (pool) uracil from E.coli K12 3300 (pBR322). Bacteria were grown to mid logarithmic phase and then incubated with 6-^{3}-H-uracil (2uCi/ml) for 45 min to form a labelled uracil pool. Bacteria were then prepared and exposed to polymyxin B (200ug/ml) (●) or PMBN 200ug/ml (○) as described in fig 6.1. A third sample served as an untreated control (□). Samples were removed at the time indicated and processed as described in the text.

Text cut off in original

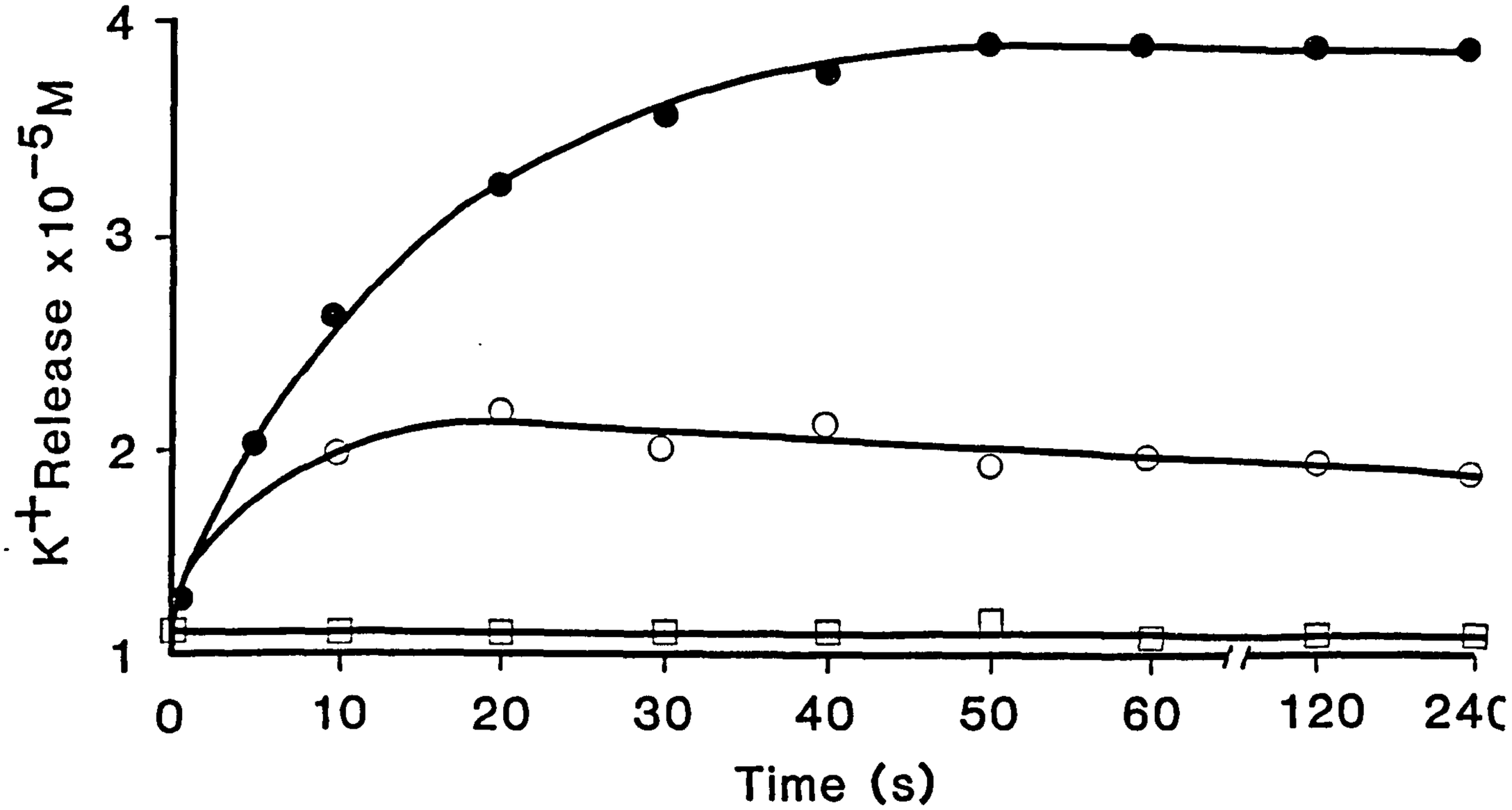
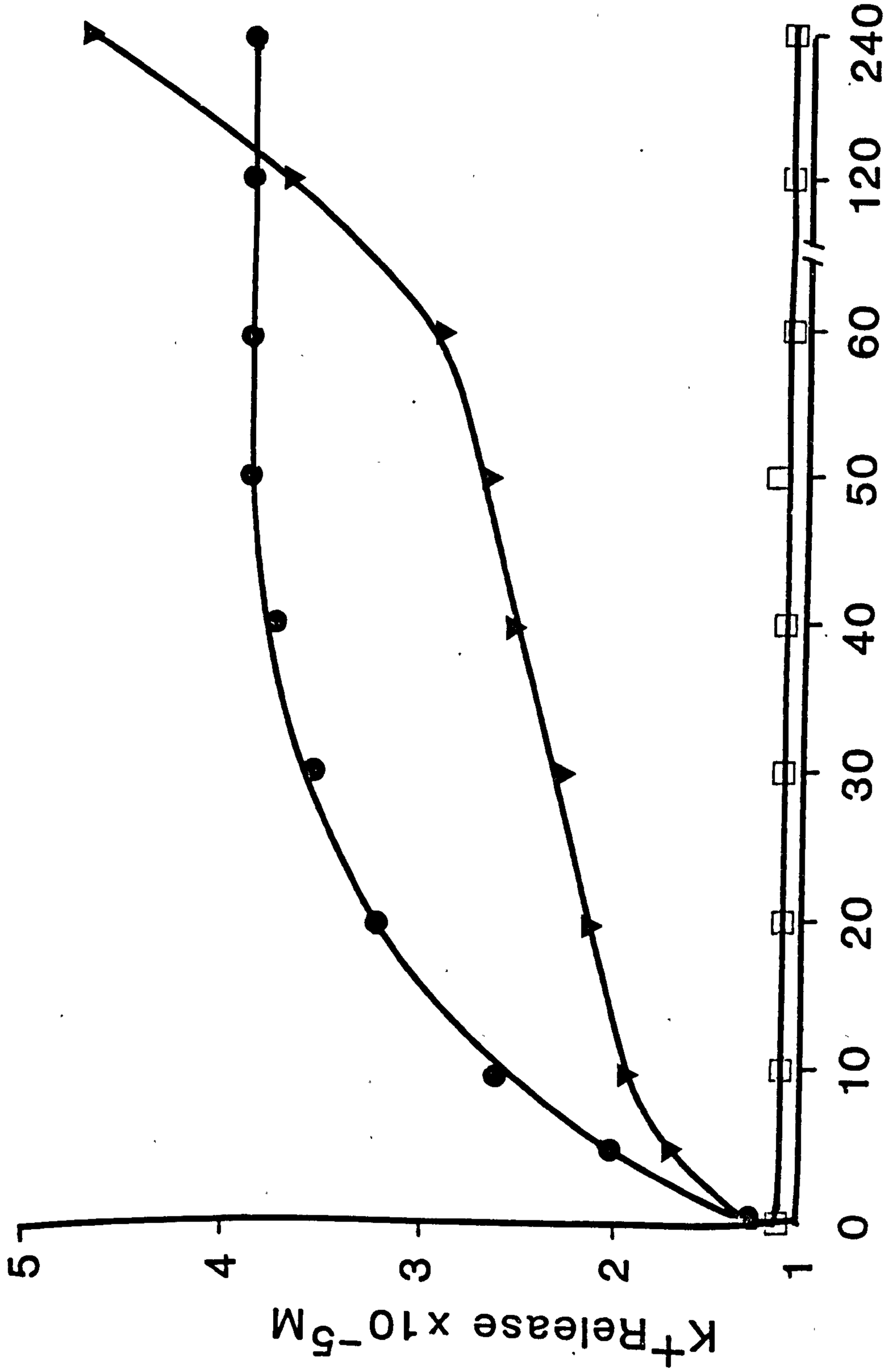


Fig 6.4 Release of potassium ions from E.coli K12 3300 (pBR322). Bacteria were grown, prepared and exposed to polymyxin B (200ug/ml) (●) or PMBN (200ug/ml) (○) as descibed in the text. A third sample served as an untreated control (□).

Thus chlorhexidine treatment elicited a response from cells similar to that obtained with polymyxin B (fig 6.5).

Fig 6.5 Release of potassium ions from E.coli K12 3300 (pBR322). Bacteria were grown, prepared and exposed to polymyxin B (200ug/ml) (●) or chlorhexidine (200ug/ml) (▼) as described in the text. A third sample served as an untreatment control (□).


240
120
60
50
40
30
20
10
0
Time (s)
5
4
3
2
1
K+Release x10-5M

DISCUSSION

Although the ability of polymyxin B to cause loss of small molecules and K^+ from Gram-negative bacteria is well documented (Storm et al., 1977; Gale et al., 1981 and Lannigan & Bryan, 1985), this is the first report that PMBN has similar properties. The capacity of both compounds to produce these changes probably reflects direct interaction with the inner (cytoplasmic) membrane of the cell envelope, because both molecules bind to cytoplasmic membranes from Gram- negative bacteria (Storm et al., 1977 and Vaara & Viljanen, 1985). However, since both compounds produce outer membrane damage (Storm et al., 1977; Gale et al., 1981 and Nikaido & Vaara, 1985); it seems probable that they both promote their own access to the inner membrane by first binding to and then disrupting the outer membrane. Indeed, this suggestion has already been specifically advanced for polymyxin B (Nikaido & Vaara, 1985).

Polymyxin B and PMBN both cause leakage of amino acids, uracil and K^+ from E.coli. Since PMBN has virtually no antibacterial activity, leakage of these cytoplasmic constituents cannot in itself be lethal. Others have also noted a lack of correlation between leakage of cytoplasmic constituents and killing in Gram-negative bacteria. For instance, Britten & McClure, (1962) found that osmotic shock reduces the amino acid pool of E.coli by about 50% without affecting protein synthesis and that other treatments which do not result in cell death may nevertheless cause complete pool

loss. Both polymyxin B and chlorhexidine at comparable concentrations, induce a rapid loss of K^+ within the first minute of exposure to cells. However, it is unlikely that potassium leakage is directly responsible for the killing of the cell. Indeed, several authors (Chen et al., 1978 and Lannigan & Bryan, 1985) have been unable to establish irrefutably that K^+ leakage is related to the killing of bacteria. Thus rapid loss of small molecules probably reflects the permeability changes caused by initial contact between the antibiotic and the inner membrane (Lannigan & Bryan, 1985).

Polymyxin B or PMBN treatment results in loss of proteins from E.coli. Those released by PMBN are derived solely from the periplasm (see Chapter 4) whereas polymyxin B induces release from both the periplasmic region and the cytoplasm (Chapter 4 and Cerny & Teuber, 1971, 1972). Both polymyxin B and PMBN promote release of small molecules and K^+ from the cell (see above). However, the integrity of the cytoplasmic membrane is only sufficiently disturbed to release proteins from the cytoplasm by polymyxin B. PMBN lacks this ability and so, despite measurable effects on the cytoplasmic membrane, fails to kill the cell.

CHAPTER 7

ULTRASTRUCTURAL CHANGES OF ESCHERICHIA COLI IN RESPONSE TO POLYMYXIN B NONAPEPTIDE OR POLYMYXIN B

INTRODUCTION

The previous chapter described the interaction of polymyxin B and PMBN with the cytoplasmic (inner) membrane. Both compounds disturbed cytoplasmic membrane integrity, reflected by loss of free amino acids, uracil and K^+ from E.coli, and for polymyxin B leakage of cytoplasmic proteins. The polymyxins also cause disorganisation of the outer membrane leading to loss of periplasmic proteins (see Chapter 4) and concomitant changes in the permeability towards hydrophobic agents (see Chapter 5; Vaara & Vaara, 1983a, b & c and Lam et al., 1986). The disorganisation induced by polymyxins may produce morphological changes in the cell envelope detectable by microscopy. Vaara & Vaara, (1983b) used transmission electron microscopy to examine bacteria for ultrastructural damage following PMBN treatment. They demonstrated PMBN-induced thin, finger-like projections on the surface of S.typhimurium.

In the present chapter, the effects of PMBN on the ultrastructure of E.coli have been examined in more detail than in Vaara & Vaara's studies by using the high resolution techniques of both transmission and scanning electron microscopy. Both are useful techniques for investigating the fine structure of bacterial cells. Scanning electron microscopy (SEM) has been used primarily in this study to examine the surface topography of bacteria, whereas fine structural detail has been provided by transmission electron microscopy (TEM). The resolution of TEM is greater than SEM, but the images produced are only two-dimensional. Although the

magnification obtained with SEM is less than that of TEM the bacteria are viewed as three-dimensional structures. In addition, processing of specimens for SEM is less disruptive to cells than for TEM (Holt & Beveridge, 1982).

Since the morphological response of E.coli and other Gram-negative cells to polymyxin B has been well documented (Wahn et al., 1968; Koike et al., 1969; Schindler & Teuber, 1975; Lounatmaa et al., 1976 and Storm et al., 1977), the structural changes in cells after exposure to PMBN have been compared to polymyxin B as a reference control.

RESULTS

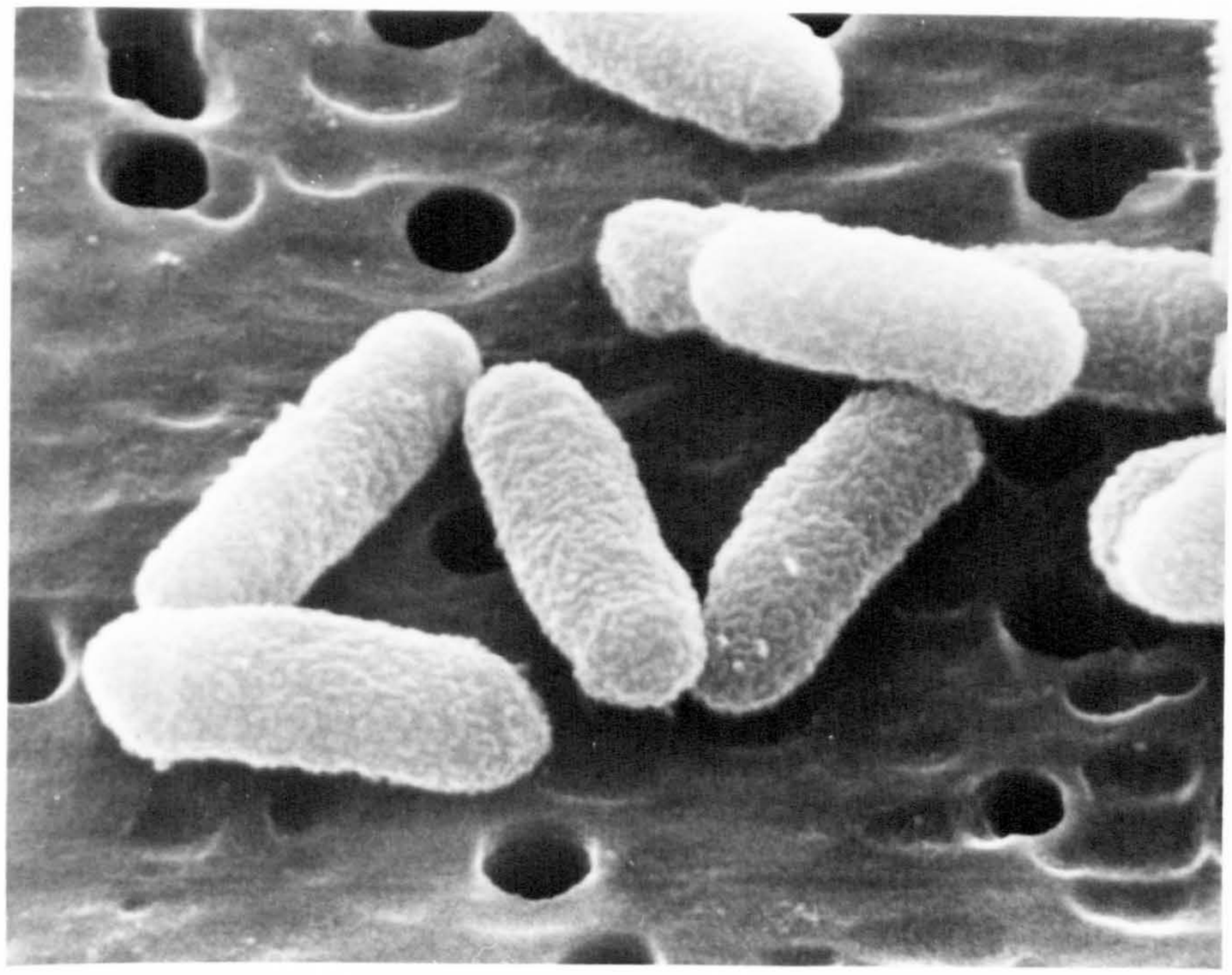
7.1 ELECTRON MICROSCOPY

7.1.1 Scanning electron microscopy (SEM)

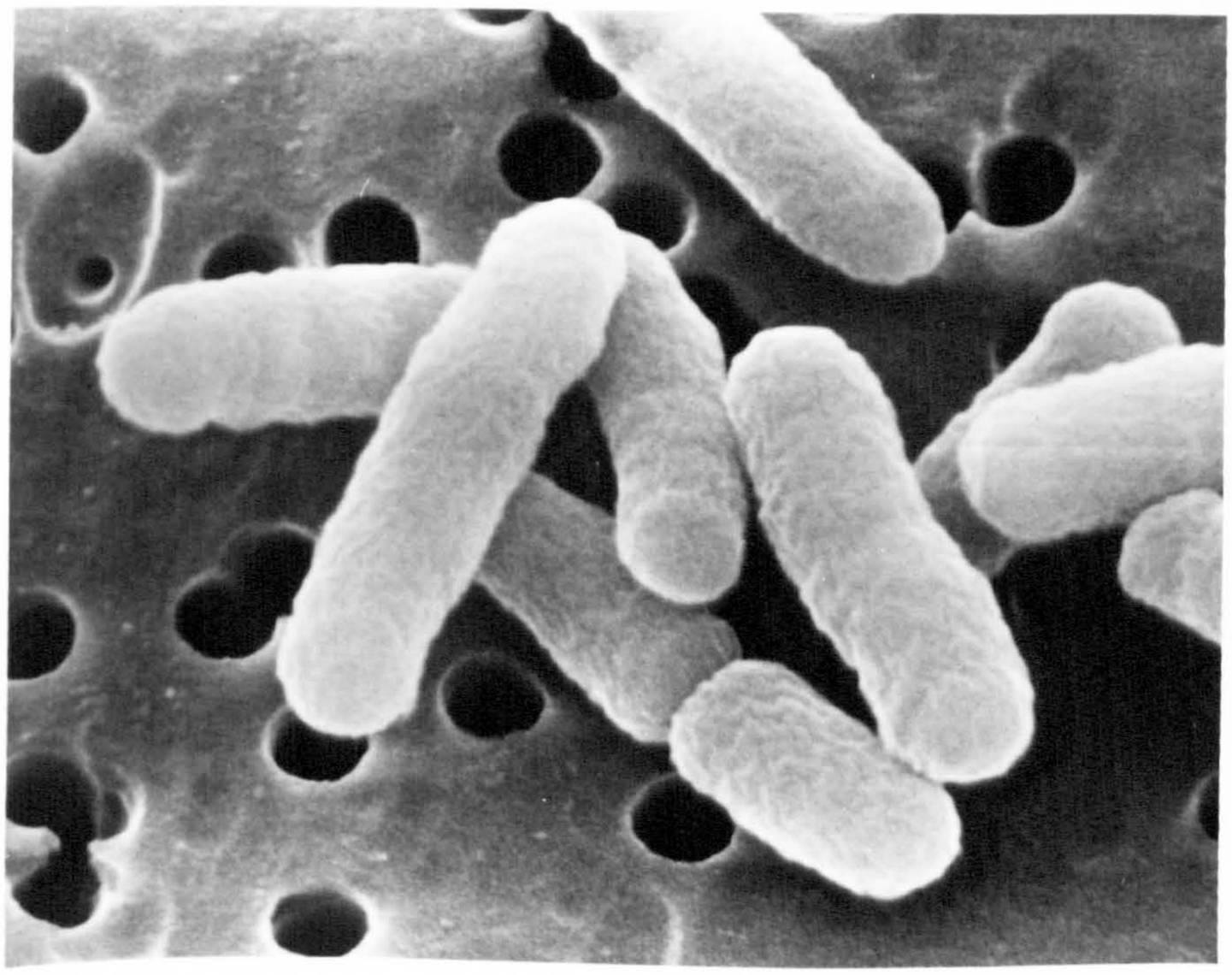
Cells of E.coli K12 3300 (pBR322) were grown, harvested and resuspended to a density of 10^{10} bacteria per ml of phosphate buffer. Bacteria were then treated with PMBN or polymyxin B (200ug/ml) for 1 hour and together with untreated controls prepared for SEM as described in Chapter 2. Bacteria (10^{10} cells per ml) exposed to either PMBN or polymyxin B for 60 min exhibited no gross change in cell shape (fig 7.1a and b) compared to controls when examined by SEM (fig 7.1c). However, the outer surfaces of cells which had been treated with polymyxin B (fig 7.1a) demonstrated a greater degree of irregularity than PMBN-treated (fig 7.1b) or untreated (control) bacteria (fig 7.1c). No evidence for cell lysis or release of particles was noted.

7.1.2 Transmission electron microscopy (TEM)

Cells of E.coli K12 3300 (pBR322) were treated with polymyxin B or PMBN as above and prepared for TEM as described in Chapter 2. Preliminary experiments were conducted to define those conditions that minimised plasmolysis of cells caused by inadequate penetration of fixative (data not shown). This allowed the characteristic triple-layered structure of the Gram-negative cell envelope to be observed. Exposure of bacteria to either polymyxin B or PMBN for 60 min led to



a



b

Fig 7.1a and b Cells (10^{10}/ml) treated with 200ug/ml of a) polymyxin B or b) PMBN for 60 min (Magnification x 28,800).

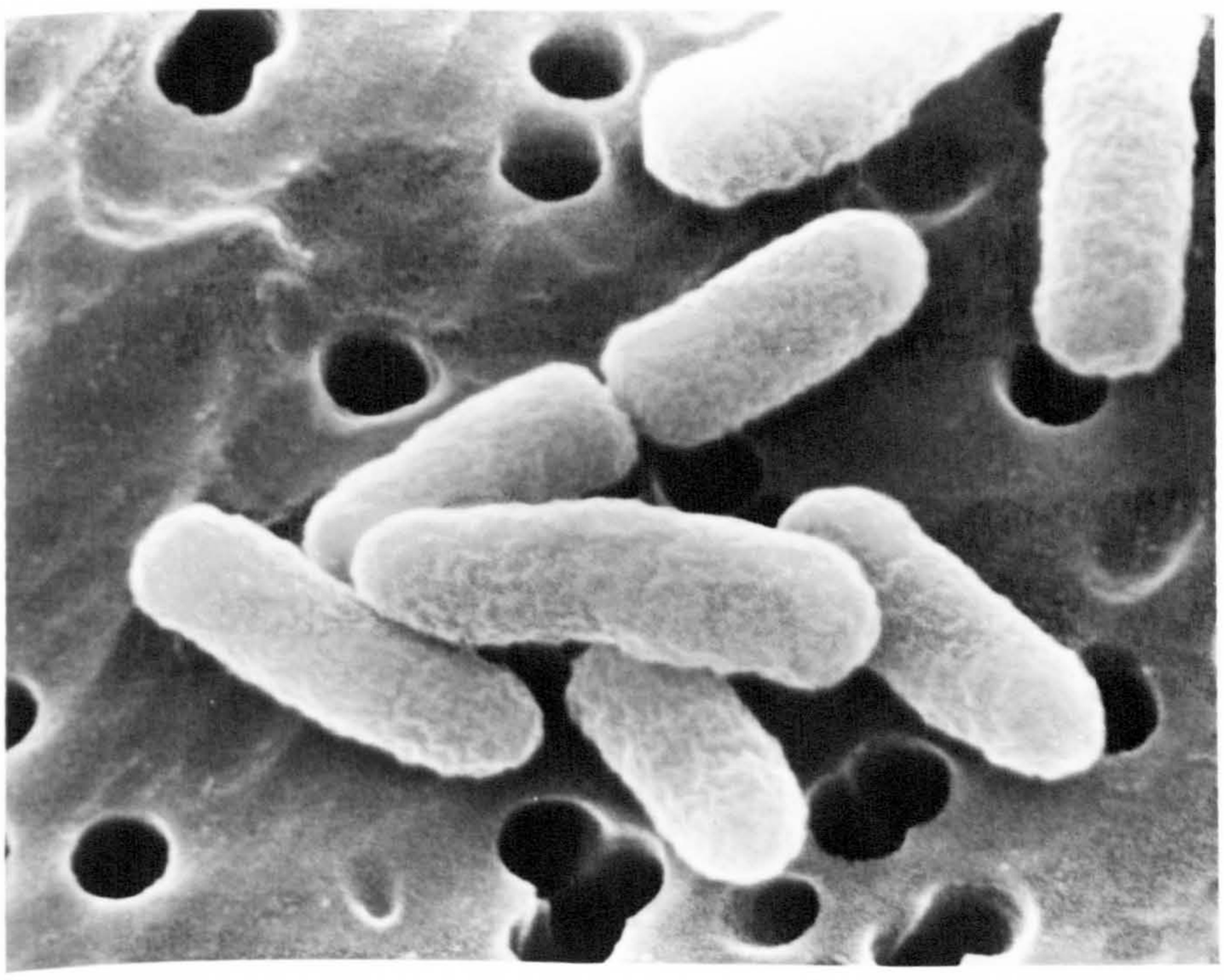


Fig 7.1c Untreated cells (10^{10}/ml) of E.coli K12 3300 (pBR322) (Magnification x 28,800).

distinct morphological changes. Polymyxin B caused the appearance of numerous protrusions over the entire surface of the cell (figs 7.2 and 7.3). Furthermore polymyxin treatment generally caused closer association between cells (e.g. figs 7.2 and 7.3). This effect may be related to the clumping of polymyxin-treated cells that was observed by phase contrast microscopy (data not shown). The response of cells to PMBN after 60 min was different, fewer protrusions were observed than with polymyxin B and these were longer, well-defined, finger-like projections (figs 7.4 and 7.5), rather than the less clearly defined protrusions seen with polymyxin B. PMBN-treated cells failed to exhibit the pronounced clumping demonstrated by polymyxin B. In contrast to the effects observed with the polymyxins the cell surface of untreated E.coli (fig 7.6) remained undisturbed. The results described above relate to samples exposed to polymyxins for 60 min. At early time points following treatment (15 min), PMBN induced vesicular structures which were apparently closely associated with the cell surface (fig 7.8). In contrast polymyxin B initially (15 min sample) developed small folds on the surface (fig 7.7) which were similar to those described by Lounatmaa et al., (1976). Both their number and size increased with time. This was consistent with the findings of these authors who showed that the response of cells to polymyxins depends on a number of factors e.g. the concentration of antibiotic and time of exposure.

The experiments just described employed conditions identical to those used in biochemical investigations of PMBN and polymyxin B presented in Chapter 4. Specifically,

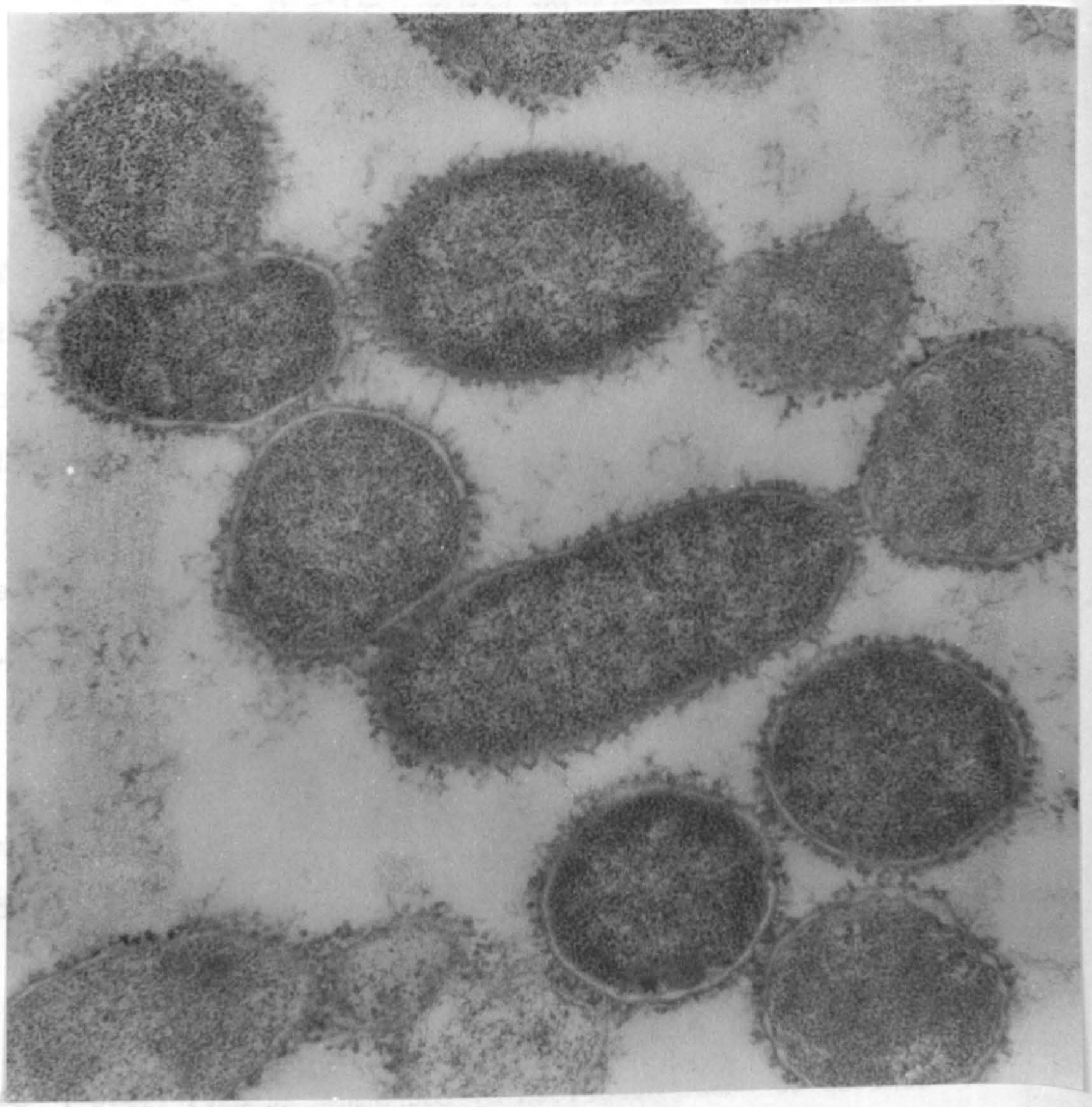


Fig 7.2 Cells (10^{10}/ml) treated with 200ug/ml of polymyxin B for 60 min (Magnification approx. x 52,000).

treatment of bacteria (10^{10}/ml) with PMBN for 60 min at 37^{0}C did not kill the organisms. However, when smaller numbers of bacteria (10^{9} cells/ml) were tested with the same concentration of PMBN (200ug/ml) as previously employed, more severe effects on the morphology of the cells were observed. A higher proportion of these cells exhibited nuclear dense material and contraction of the cytoplasm (fig 7.10), indicating cellular death. Polymyxin B-treated cells also demonstrated extensive damage when tested at the lower cell density (fig 7.9). Moreover, when cell numbers were further reduced to approximately 10^{6} cells per ml, the structural damage caused by both PMBN and polymyxin B was severe enough to be clearly seen using SEM (figs 7.11a and b). Exposure to polymyxin B produced extensive protrusions or blebs on the surface of E.coli and some of this particulate material appeared to be released from the cells, together with loss of the general structural integrity of the cell (fig 7.11a). The changes caused by PMBN were less severe but also appeared to involve the formation of large blebs on the cell surface (fig 7.11b). In contrast, the untreated E.coli cells (fig 7.11c) had a much smoother surface than those treated with either polymyxin B or PMBN and separate particles were rarely seen.

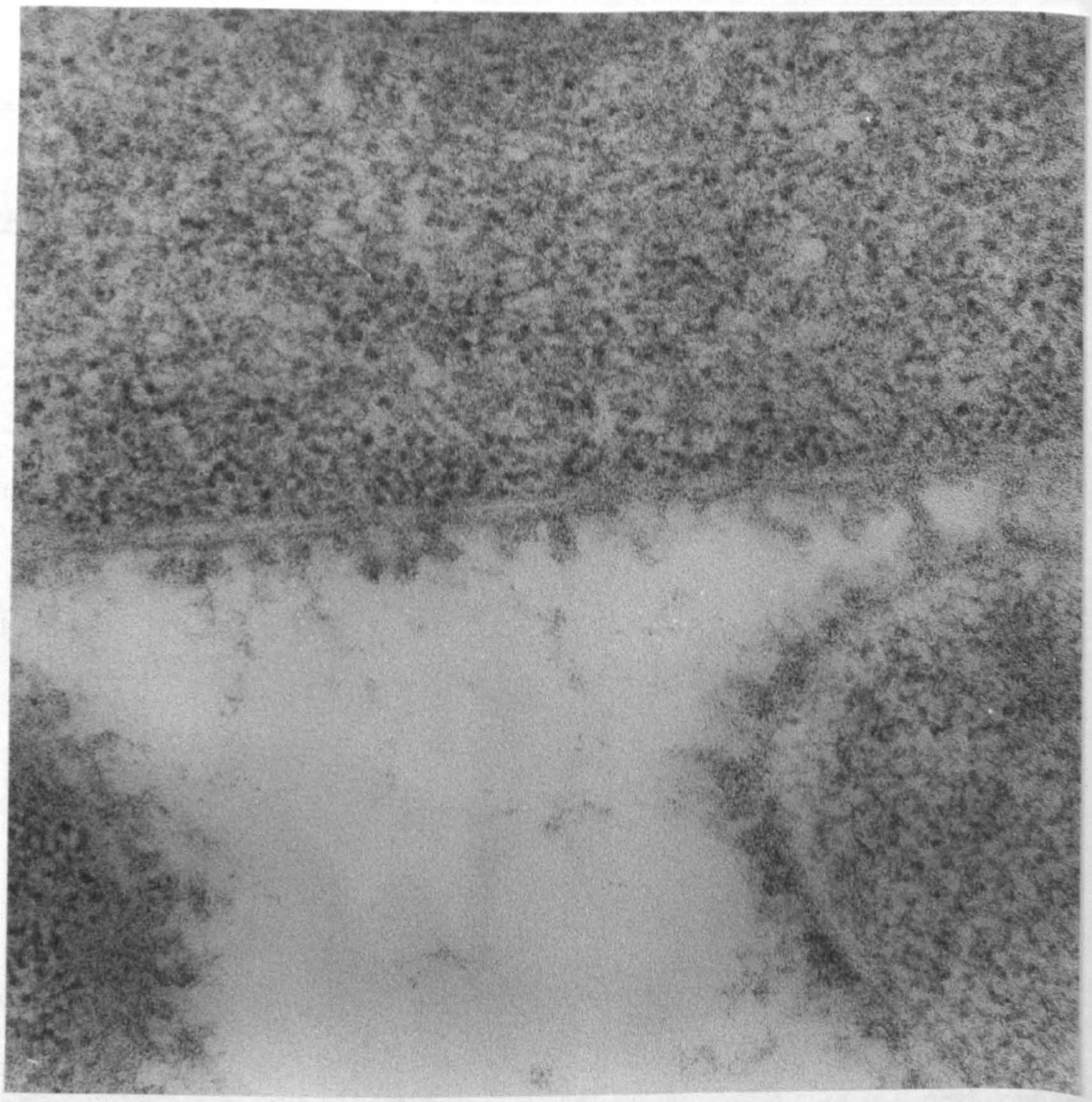


Fig 7.3 Higher magnification of cells from fig 7.2 treated with 200ug/ml of polymyxin B for 60 min (Magnification approx. x 170,000).

7.2 EFFECT OF PMBN ON VIABILITY OF CELL SUSPENSIONS CONTAINING LESS THAN 10^{10}/ML BACTERIA

As seen above, the exposure of a progressively decreasing number of cells to a fixed concentration of PMBN increased the degree of cellular damage. This prompted an experiment to investigate the viability of PMBN-treated E.coli at lower bacterial numbers than previously employed. This showed that 200ug/ml of PMBN, which had consistently failed to kill cells at concentrations of 10^{10}/ml, exhibited considerable potency against smaller cell numbers. At 10^9 cells per ml, PMBN killed 95% of the culture and this was further reflected by a general increase in PMBN potency at lower cell densities (data not shown). The bactericidal effects of PMBN on suspensions at 10^9 cells per ml were consistent with the morphological evidence of severe cellular damage shown in fig 7.10.

Fig 7.4 Cells (10^{10}/ml) treated with 200ug/ml of PMBN for 60 min (Magnification approx. x 84,500).

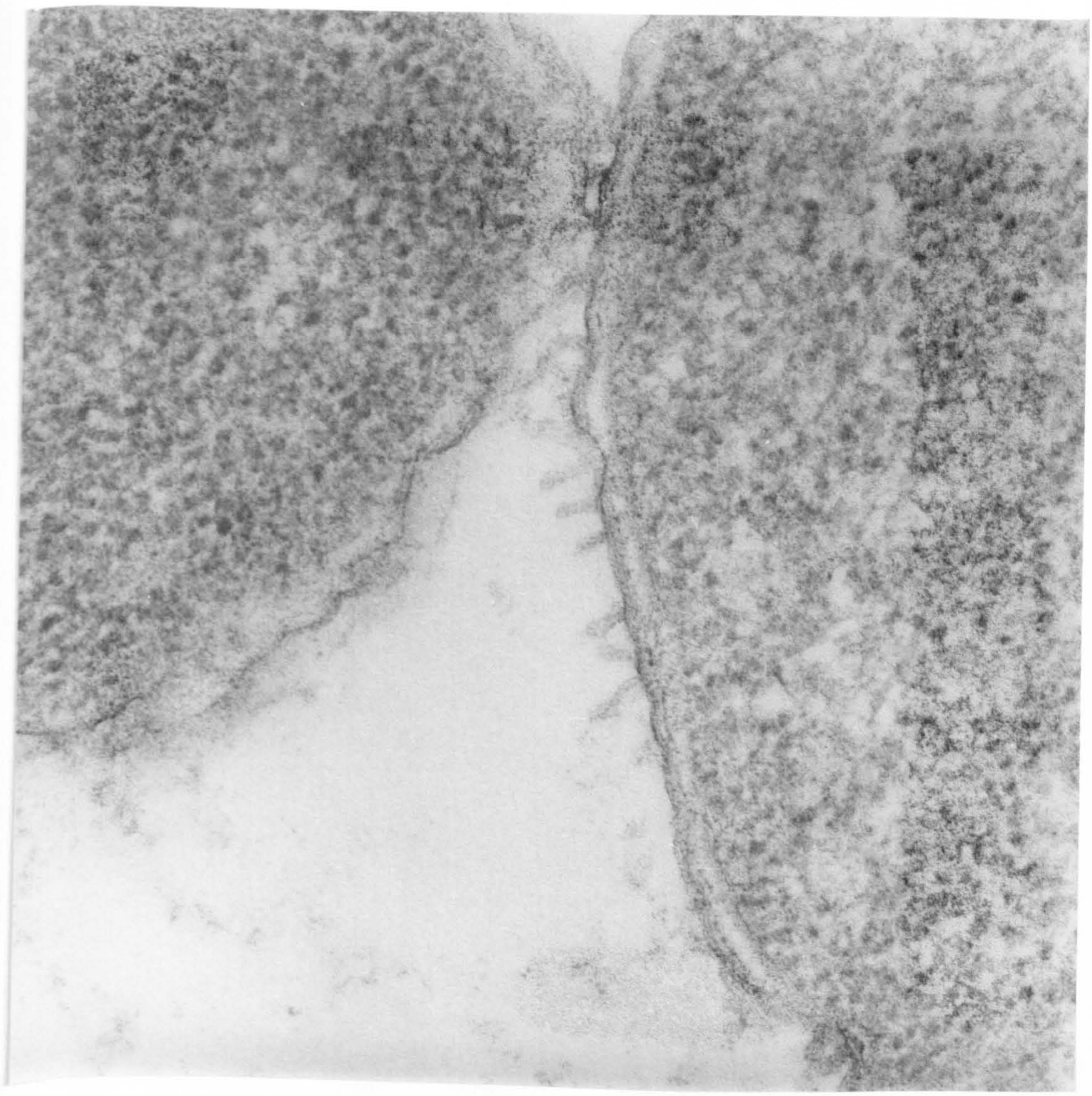


Fig 7.5 Higher magnification of cells from fig 7.4 treated with 200ug/ml of PMBN for 60 min (Magnification approx. x 170,000).

Fig 7.6 Untreated cells 10^{10}/ml of E.coli K12 3300 (pBR322) (Magnification approx. x 52,000).

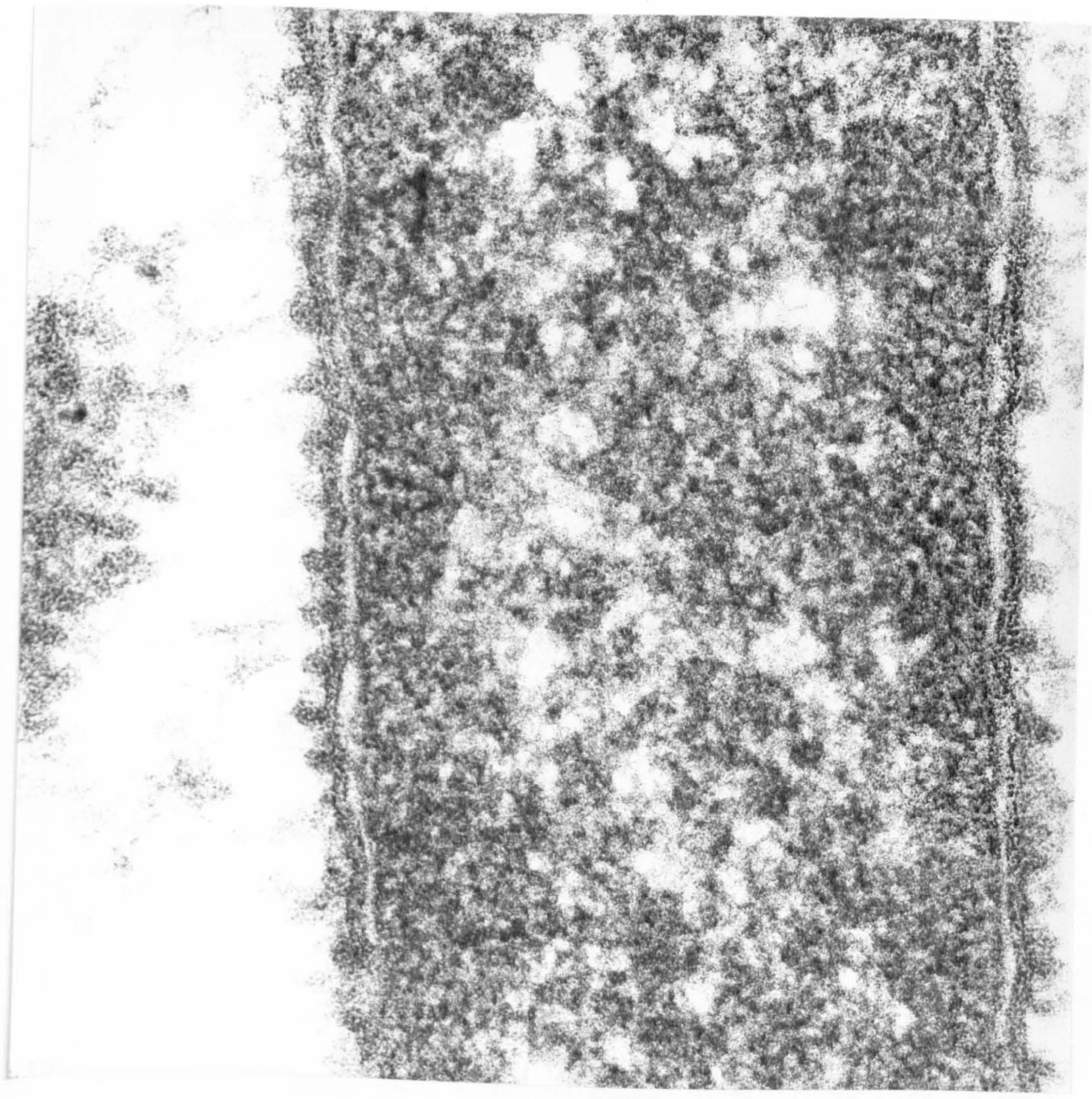


Fig 7.7 Detail of ultrastructure of cells treated with 200ug/ml of polymyxin B for 15 min (Magnification approx. x 170,000).

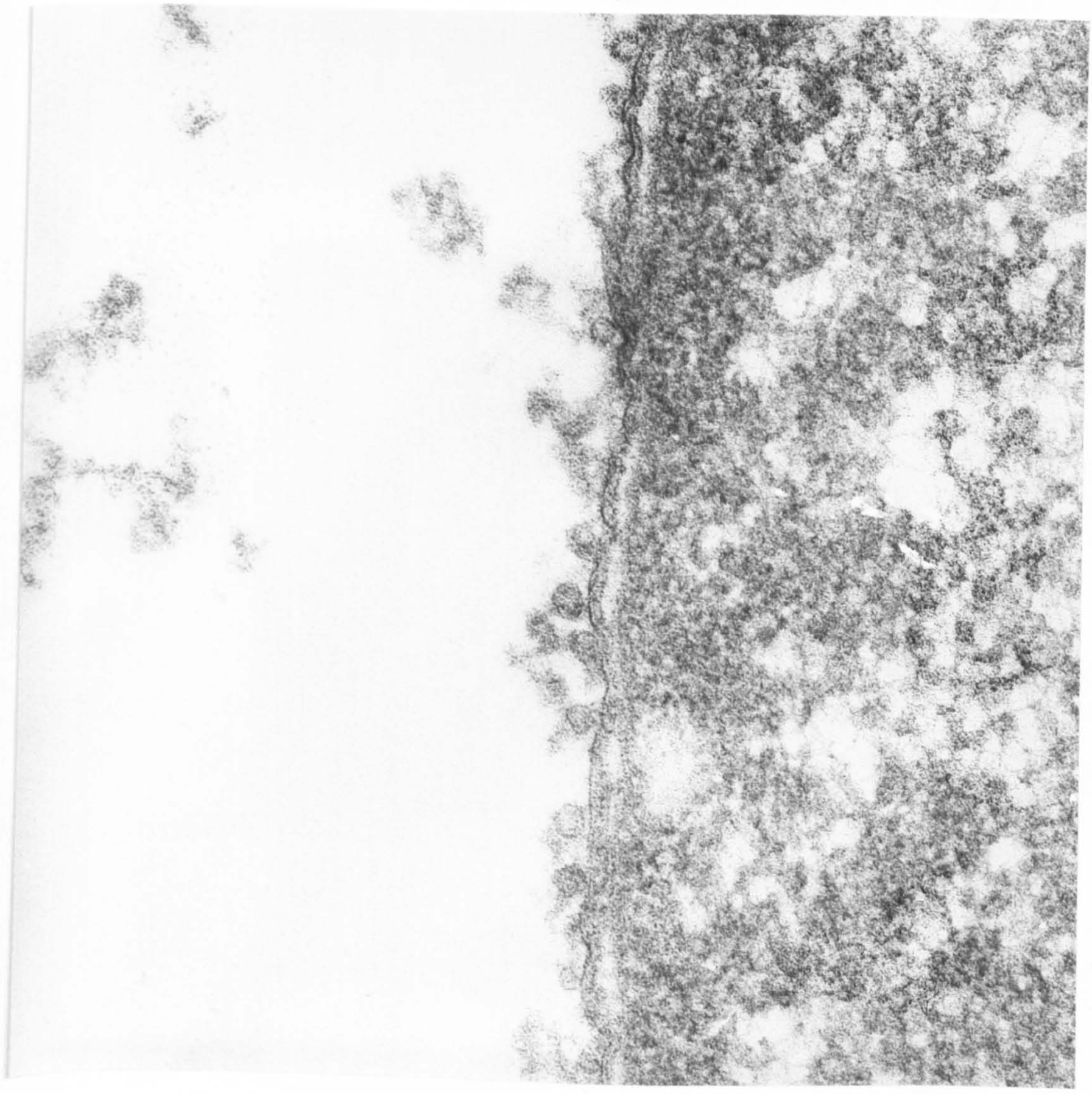


Fig 7.8 Detail of ultrastructure of cells treated with 200ug/ml of PMBN for 15 min (Magnification approx. x 170,000).

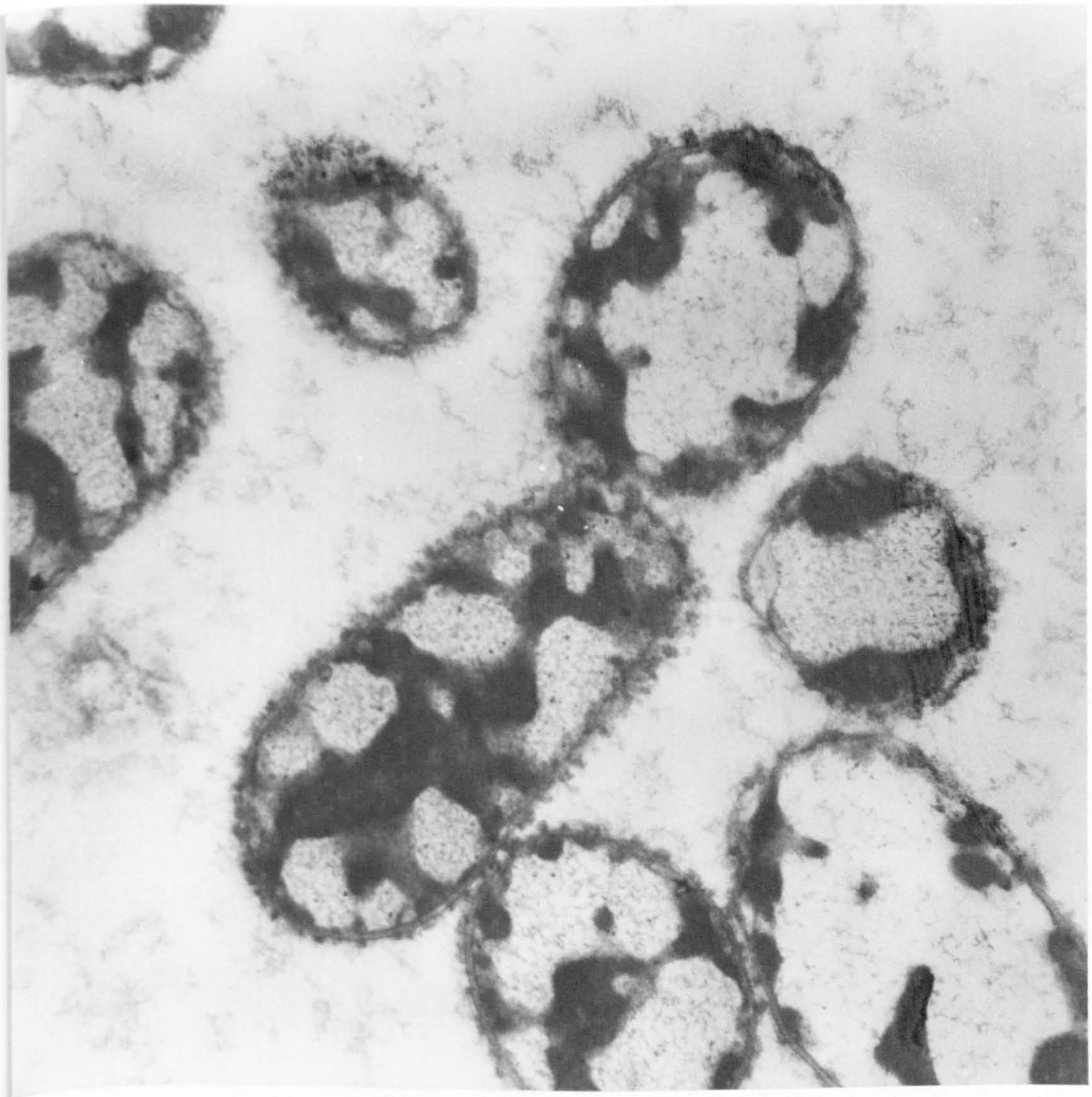


Fig 7.9 Cells (10^9/ml) treated with 200ug/ml of polymyxin B for 60 min (Magnification approx. x 52,000). Note brightening of the nuclear area.

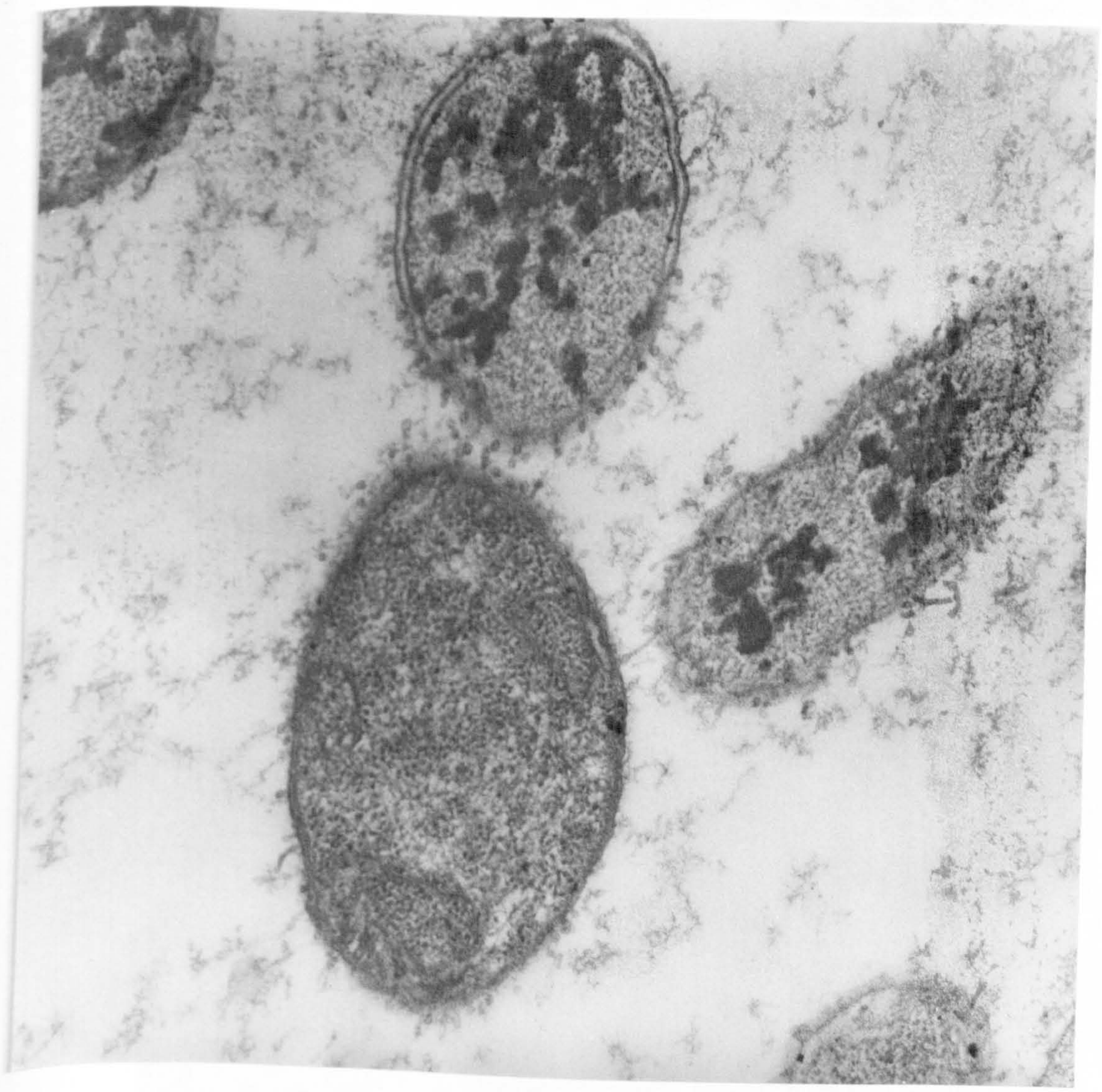
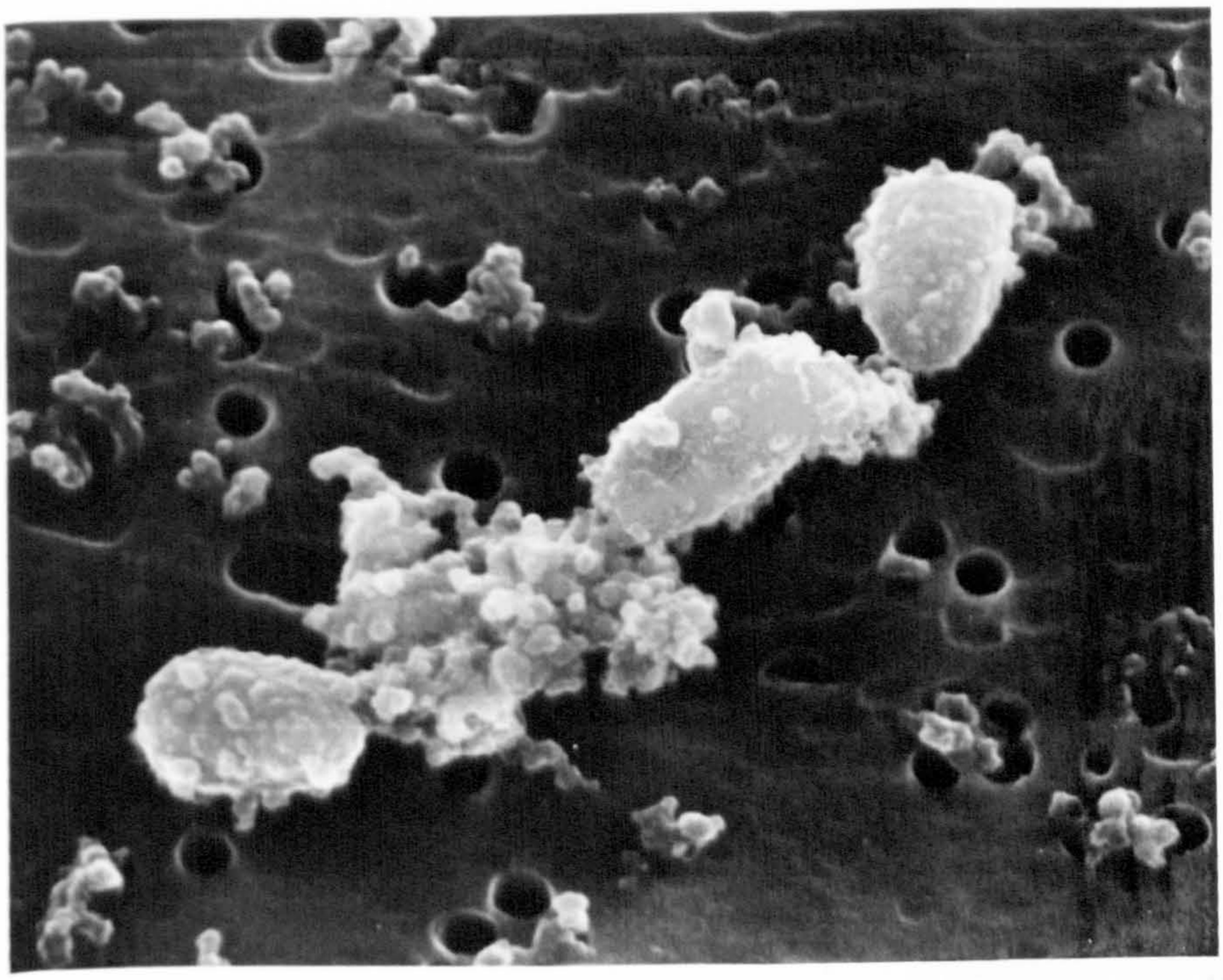
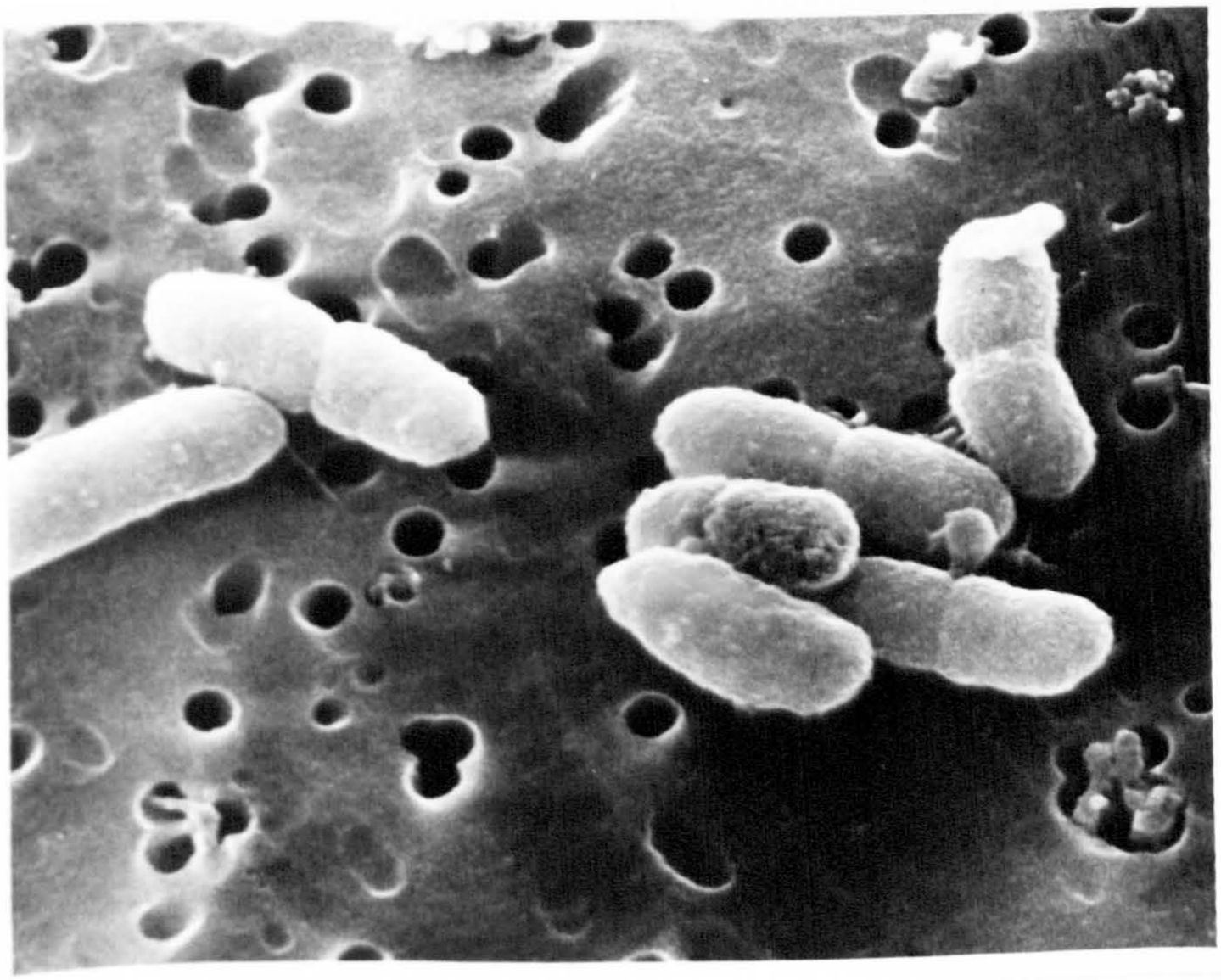


Fig 7.10 Cells (10^9/ml) treated with 200ug/ml of PMBN for 60 min (Magnification x 63,750). Note areas of nuclear dense material.



a



b

Fig 7.11a and b Cells (approx 10^6/ml) treated with 200ug/ml of a) polymyxin B or b) PMBN for 60 min (Magnification x 14,400). Note particulate material.

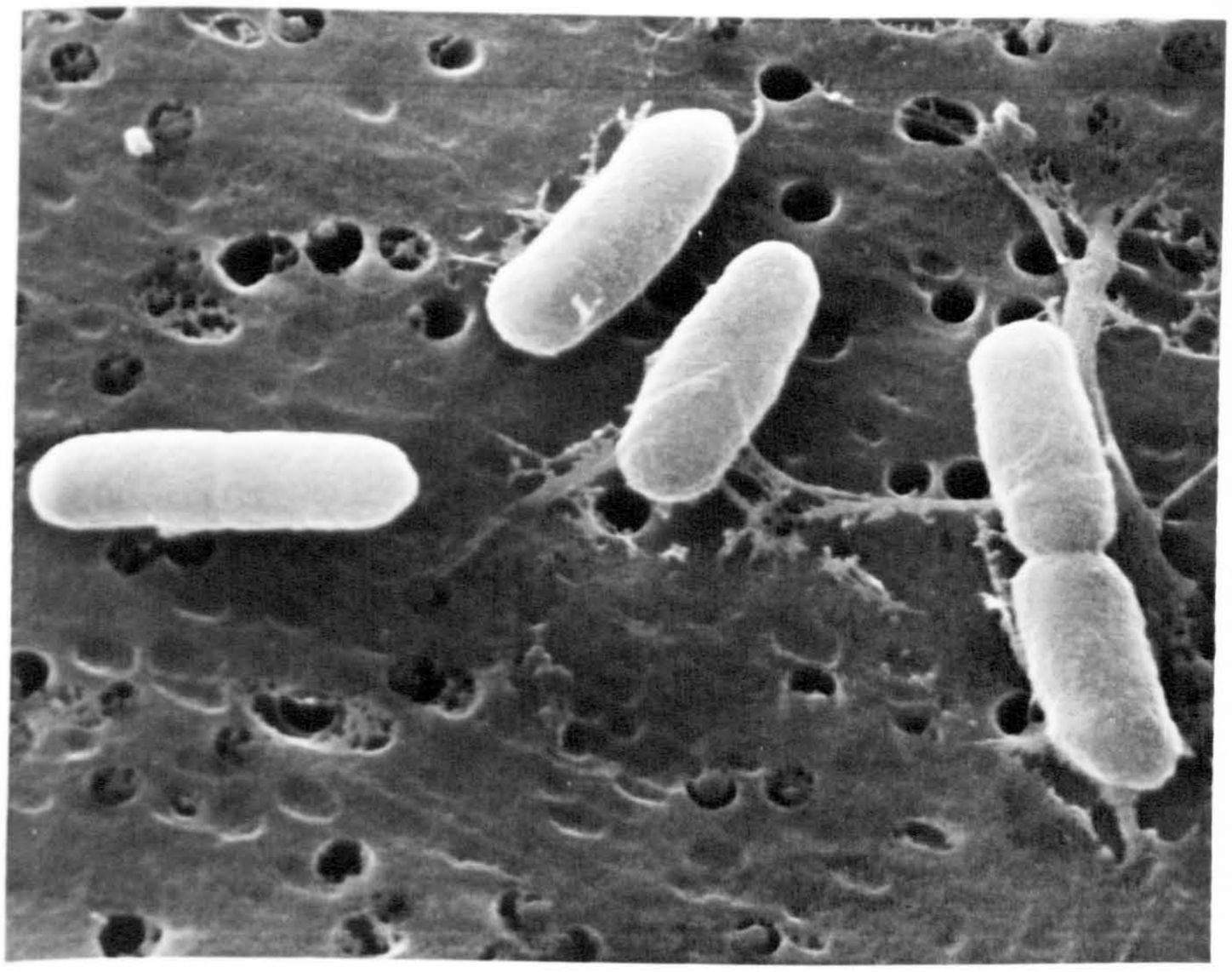


Fig 7.11c Untreated cells (approx 10^6/ml) of E.coli K12 3300 (pBR322) (Magnification x 14,400).

DISCUSSION

This chapter has described the morphological responses of E.coli to the polymyxins. Experiments were designed to duplicate the conditions previously employed to investigate the biochemical effects of the polymyxins (see Chapters 3 - 6). Under conditions which had promoted the leakage of periplasmic proteins and soluble cytoplasmic components, both polymyxin B and PMBN caused distinct morphological changes in the outer layers of E.coli. It would seem likely therefore, that the observed morphological disruption of the outer membrane accounts directly for the loss of periplasmic proteins. The image of the cytoplasmic membrane however is not easily visualised by either SEM or TEM. Nevertheless, no gross morphological alterations were observed despite biochemical evidence that the inner membrane was perturbed.

The findings presented here for polymyxin B are in general agreement with earlier work concerning the effects of polymyxins on the ultrastructure of Gram-negative cells (Chapman, 1962; Suganuma et al., 1968; Wahn et al., 1968; Koike et al., 1969; Koike & Iida, 1971 and Lounatmaa et al., 1976). The responses to polymyxin B of the E.coli strain used here and a strain of S.typhimurium described by Lounatmaa et al., (1976) were remarkably similar. Viewed by TEM, both organisms produced small wide flat projections following treatment with polymyxin B.

The projections induced by polymyxin B clearly involve degradation of the cell envelope outer membrane. They appear

to be extensions of this structure, which nevertheless remains continuous throughout. Lounatmaa et al., (1976) have studied the protrusions produced by polymyxin B in S.typhimurium. Since they had no distinct morphology, it was concluded that these projections might simply be lipid micelles, perhaps representing LPS and other outer membrane components. In contrast to polymyxin B, PMBN promoted long finger-like, but less numerous, projections on the surface of E.coli consistent with the response of S.typhimurium to PMBN previously described by Vaara & Vaara, (1983b).

The separate morphological responses produced by these compounds probably reflects the difference in their structures, which in turn results in different modes of binding to the envelope. Polymyxin B intercalates with the LPS causing massive expansion of the surface area and forcing the membrane into numerous folds (Lounatmaa et al., 1976). PMBN shares the same binding site (acidic LPS) but has a lower affinity for it than polymyxin B (Vaara & Viljanen, 1985). The higher affinity of polymyxin B must be due to its fatty acid component which is thought to interact hydrophobically with the lipid A of the LPS (Vaara & Viljanen, 1985). In addition, the different structural changes observed after polymyxin B and PMBN treatment could reflect the different effects of the compounds on general cell metabolism. Metabolic processes stop rapidly as a result of the lethal effects of polymyxin B, whereas limited metabolism, in PMBN-treated cells, might continue in phosphate buffer thus allowing further cell surface extension.

Release of particulate material was only observed under the electron microscope when low numbers of cells were exposed to polymyxin B or to PMBN (figs 7.11a & b). Studies with the polymyxin analogue, EM49 show that this agent is capable of releasing particles from E.coli, identified as outer membrane fragments (Rosenthal et al., 1976). However there is no biochemical evidence from this study or that of other workers (Cerny & Teuber, 1972; Storm et al., 1977; and Vaara & Vaara, 1981, 1983b) that polymyxin B or PMBN actually release outer membrane material from Gram-negative bacteria.

Finally, the observation that exposure of a decreasing number of cells to a fixed concentration of PMBN or polymyxin B increases the degree of cellular damage is interesting. It suggests that polymyxin B or PMBN cause less extensive disruption to large numbers of cells because their high surface area, with accessible LPS binding sites, exhausts all the available polymyxins.

CHAPTER 8

GENERAL DISCUSSION

This thesis has presented microbiological, biochemical and morphological studies on the mode of action of polymyxin B and its derivative PMBN. A further aspect of the studies has been to reveal details of the molecular organisation of the Gram-negative bacterial cell envelope.

The investigations presented here have confirmed that removal of the N-terminal diaminobutyric acid residue with attached fatty acid, from polymyxin B, produces a derivative (PMBN) with poor antibacterial activity compared to the parent compound (Chapter 3). Furthermore, it was also confirmed that PMBN sensitizes E.coli to a number of hydrophobic antibiotics (Chapter 3). Investigations of this phenomenum by Vaara & Vaara (1983a and c) suggested that sensitization of Gram-negative bacteria by PMBN occurred at the level of the cell envelope. This prompted them to study a number of biochemical factors that indicate membrane disruption. Their results showed that although PMBN could be classified as an agent capable of interacting with the outer membrane (Vaara & Viljanen, 1985), it did not provoke the major disruption of membrane integrity associated with polymyxin B. The results presented in this thesis confirm the ability of polymyxin B to release both periplasmic and cytoplasmic proteins from cells. This is a clear indication that disruption of both outer and inner membrane integrity has occurred (Chapter 4 and Cerny & Teuber, 1971 and 2). However, despite reports by Vaara & Vaara that PMBN modifies the permeability of the outer membrane and even causes ultrastructural damage, these workers were unable to demonstrate leakage of proteins from treated cells. In

contrast however, the results in Chapter 4 have clearly shown that PMBN releases periplasmic proteins.

Agents able to disrupt the integrity of Gram-negative outer membranes can be classified into two general groups: i) those that release periplasmic proteins and rapidly sensitize cells to hydrophobic antimicrobial agents, and ii) those that do not cause periplasmic leakage and lead only to slow sensitization to hydrophobic antibiotics (Nikaido & Vaara 1985). PMBN exerts a rapid sensitizing action and would therefore appear to fall into the first group. Its apparent inability to release periplasmic proteins previously precluded this classification, but the data presented in Chapter 4 resolves the dilemma; i.e. PMBN, like other compounds which cause rapid sensitization to hydrophobic antibiotics, releases periplasmic proteins.

It is paradoxical that although polymyxin B and PMBN have strong affinities for the outer membrane (Vaara & Viljanen, 1985) and affect its integrity, there are no reports of the release of proteins or other components from the outer membrane following treatment (Cerny & Teuber, 1972; Storm et al., 1977 and Vaara & Vaara, 1981 and 1983a, b & c). This study has established that proteins released by PMBN were derived exclusively from the periplasm. PMBN released less protein than polymyxin B although the individual proteins released, differed. The release of different proteins from the periplasmic region of the cell suggests that polymyxin B and PMBN act differently on the cell envelope. This may be due to the nature of the interaction between polymyxin B or PMBN and

the LPS of the outer membrane. Vaara & Viljanen (1985) reported competition data to show that polymyxin B has a higher affinity for the acidic LPS binding site than PMBN. The binding affinities of the compounds for this site are probably reflections of differences in their chemical structure. The higher affinity of polymyxin B is probably due to its fatty acid component, which is thought to interact hydrophobically with lipid A of LPS (Vaara & Viljanen, 1985). Therefore, the release of different proteins by the two polymyxins could be due indirectly to the different modes of binding of the compounds to LPS. These effects may induce topographical changes in the organisation of the outer membrane allowing the release of specific periplasmic proteins.

Another important observation relating to the binding of polymyxins to LPS is their ability to displace magnesium ions from surface sites such as phosphate on LPS (Nicas & Hancock, 1980 and Hancock, 1981). Polycationic compounds such as the polymyxins attack the divalent cross-bridges between LPS molecules. Competition by polymyxin B for cation binding sites displaces magnesium ions and increases the permeability of the outer membrane with concomitant loss of periplasmic proteins (Cerny & Teuber, 1971). The permeability changes noted with EDTA (Chapter 5) also appear to be due to disturbances of the same site. However, EDTA is a strong chelating agent and therefore the mechanism which leads ultimately to permeability changes, appears to be chelation rather than competition for the site. The lytic effects of polymyxin B can be inhibited by high concentrations of competing cations (Newton, 1953) and

studies by Brown (1975) and Nicas & Hancock (1980) have supported the contention that competition for the cation binding site in the core region of the LPS, is an important factor in polymyxin activity. Although Storm et al. (1977) have suggested that polymyxin B might bind relatively non-selectively to both KDO and phosphate groups of the LPS, the detailed work of Schindler & Osborn (1979) with S.typhimurium LPS has demonstrated novel high affinity divalent cation binding sites. These sites appear to be formed by the three highly negative KDO residues of the LPS molecule (see Chapter 1) and are possibly the molecular site of competition between divalent cations and polymyxin B in the outer membrane. A report that binding of PMBN to LPS can also be inhibited by high concentrations of competing divalent cations (Vaara & Viljanen, 1985) supports the contention of a common binding site on the outer membrane for both polymyxin B and PMBN. Recent studies by Peterson et al. (1985) have investigated the ability of polymyxin B and PMBN to alter the packing arrangement of bacterial LPS. Using electron spin resonance techniques (Coughlin et al., 1981) the ability of polycations to displace the cationic spin probe (CAT_{12}) from P.aeruginosa LPS was assessed. Binding of either PMBN or polymyxin B to the anionic groups on the LPS altered the conformation of LPS aggregate structure apparently by disrupting LPS-LPS interactions. However, although both compounds appear to bind to the same site on the outer membrane, their subsequent effects in terms of the release of individual proteins, as well as on the viability of the cell as

shown in this thesis, are different.

The release of different proteins from the periplasm has been a feature of the studies presented here and may suggest that polymyxin B and PMBN promote polypeptide release from specific compartments in the periplasm. This suggestion would depend on a more ordered arrangement and compartmentalisation of proteins in the periplasm than previously considered (see Chapter 1 and Costerton et al., 1974). However, recent investigations into the organisation of the periplasm by Hobot et al. (1984) and Brass et al. (1986) have led to a reconsideration of the nature of the 'periplasmic space'. These workers present data suggesting that the periplasm is extremely viscous and might in fact consist of a gel. Brass et al. (1986) concluded that the periplasm is a single contiguous compartment surrounding the cell. However, their data is consistent with a model that the periplasm may be divided into many small but connected compartments, with barriers restricting the area through which diffusion from compartment to compartment could occur. The release of different proteins from the periplasm described in this thesis is consistent with the model presented above although no electron microscopic evidence at present exists to suggest compartmentalisation of the periplasm (Brass et al., 1986).

Apart from binding to the outer membrane, it is clear from data presented in this thesis that both polymyxin B and PMBN are likely to interact directly with the cytoplasmic membrane. For instance, the data in Chapter 6 show that both polymyxin B and PMBN cause leakage of small molecules and potassium ions

from Gram-negative cells. The degree of damage measured by these markers probably reflects the binding of the compounds to the cytoplasmic membrane (Storm et al., 1977 and Vaara & Viljanen, 1985). The disruptive effects of polymyxin B were severe enough to allow the release of large proteins (beta-galactosidase) from the cytoplasm (Chapter 4; Cerny & Teuber, 1971 and Vaara & Vaara, 1981). The bactericidal properties of polymyxin B however, are likely to be related to other aspects of cytoplasmic membrane function that were not examined (see later).

As mentioned above and in Chapter 1, the molecular basis of polymyxin B action is poorly understood and probably involves a number of simultaneous effects on the cell. At the molecular level a number of studies have suggested that polymyxin B, being an amphipathic molecule, destabilises bilayers by interacting with both the polar and non-polar regions of lipids (Teuber & Bader, 1976). However, recent studies with model membrane systems (Theretz et al., 1984) have produced two suggestions on how polymyxin B might interact with bilayers. First that polymyxin B is adsorbed at the surface of the lipid layer with only its hydrophobic tail penetrating between the lipid molecules (Hartmann et al., 1978 and Sixl & Galla, 1982). Secondly the entire polypeptide penetrates deeply into the lipid bilayer (El Mashak & Tocanne, 1980). The importance of the fatty acid tail of polymyxin B in membrane interactions is indicated in a novel system reported by Carr & Morrison (1985). Polymyxin B induced haemolysis of

LPS-coated rabbit erythrocytes whereas PMBN had no lytic effect. Furthermore these authors showed that PMBN blocked the lytic effect of polymyxin B on the erythrocytes and suggest that although PMBN retains the capacity to bind to LPS, the presence of the terminal fatty acid of polymyxin B is critical for erythrocyte lysis.

However, despite theories concerning the molecular events leading to loss of cell integrity, it remains clear that polymyxin B promotes leakage of cytoplasmic proteins (Cerny & Teuber, 1971). These proteins may play a role in the lytic event, as release of murein hydrolases might explain why polymyxin B-treated cells rapidly lyse (White et al 1949., and Tai & Van Heyningen, 1951). Bacterial murein hydrolases (autolytic enzymes) participate in normal murein expansion by providing space and acceptor sites for new material to be condensed into the growing sacculus by murein synthetic enzymes (Holtje & Schwarz, 1985). Under normal conditions the autolytic enzymes are prevented from causing gross digestion of the murein that would lead to cell lysis. Control of these enzymes to prevent digestion of the cells own murein is obviously important. The cytoplasmic membrane is one of the important factors in providing an anatomical barrier to separate peptidoglycan from hydrolytic enzymes (Hartmann et al., 1974 and Holtje & Schwarz, 1985). Disruption of the normal anatomical barrier by polymyxin B, thereby permitting leakage of autolytic enzymes could possibly account for the bactericidal properties of the antibiotic. In E.coli, amidase B-N-acetylglucosaminidase and peptidase have been

detected (Rogers et al., 1980) and the amidase has been purified. Studies to detect the location and subsequent release of murein hydrolases in the cell envelope following polymyxin B treatment would be of interest.

Evidence for morphological damage to E.coli produced by both PMBN and polymyxin B was obtained by electron microscopy. Disturbance of the surface morphology of bacteria was consistent with the reported effects of a number of other membrane-active agents. These included the polycations, protamine and polylysine (Nikaido & Vaara, 1985), EDTA (Hancock 1984) and octapeptin (Storm et al., 1977). In addition the outer surfaces of cells can be made to 'bleb' by physical treatments. Examination of E.coli exposed to reagent grade water show outer membrane blebbing as do cells that are heat treated (Tsuchido et al., 1985). It has also been thought that morphological effects seen in electron micrographs of cells treated with membrane-active agents might be artefacts of fixation and thin sectioning of weakened membranes. However, the bacteria described in Chapter 7 were also examined by SEM, a technique that is less destructive to the cell envelope than fixation and thin sectioning. Application of this technique, together with TEM demonstrated that E.coli treated with either polymyxin B or PMBN had numerous blebs on the outermost surface, probably as a result of antibiotic intercalation into the membrane surface (see Chapter 7). Since PMBN and polymyxin B both cause disruption of the outer membrane, the fatty acyl tail associated with the latter does not appear to be a requirement for intercalation with membranes and the ensuing

development of protrusions.

Interestingly, the aminoglycoside antibiotic gentamicin is another agent which has recently been reported to cause extensive protrusions on the surface of Gram-negative cells (Martin & Beveridge, 1986). These authors have shown by a variety of electron microscopy techniques that gentamicin induced blebs when P.aeruginosa or E.coli K12 were treated. Furthermore, gentamicin appears to displace divalent cations in the outer membrane of the cells in a manner similar to polymyxin B. These authors suggest the initial interaction of gentamicin with Gram-negative cells appears to be comparable to that of the polymyxins. They were able to follow the disruption of the cell envelope caused by gentamicin in P.aeruginosa. Remarkably they were able to observe small transient holes within the outer membrane and follow the disruptive process through from outer to inner membrane. Despite the intensive examination by thin-section studies described in this study, breaks or holes in the continuity of the outer membrane of cells treated with either PMBN or polymyxin B were not revealed. Furthermore, it was not possible with polymyxin B-treated cells to discern areas of degradation of the peptidoglycan or disruption of the inner membrane related to antibiotic action. However, in their studies (Martin & Beveridge, 1986) showed that gentamicin led to release of outer membrane proteins and LPS from the cell. In contrast, I was unable to demonstrate loss of outer membrane components after polymyxin B or PMBN treatment.

Since one of the important features of PMBN is to

facilitate the penetration of antibiotics into Gram-negative bacteria, its possible value in the treatment of infections is obvious. Lam et al (1986) were the first to explore the extent to which the outer membrane disorganising activity of PMBN could be applied to the treatment of experimental infections. These studies have shown that PMBN was ineffective against mice infected systemically with E.coli. There was no protective effect against experimental septicaemia when PMBN at concentrations of up to 200mg/kg body weight was tested alone or in combination with an equivalent concentration of erythromycin (Lam et al., 1986). This was a disappointing finding since PMBN interacts synergistically with erythromycin against this strain of E.coli in vitro.

Finally, in the future, specific areas of interest concerning PMBN might be i) its effects on the interaction of bacterial adhesins with their mammalian receptors (see Chapter 1) and ii) the effects of PMBN on bacterial conjugation. Preliminary results from these laboratories show that K88-mediated adhesion of E.coli to guinea pig erythrocytes is significantly reduced by PMBN. Whether PMBN acts by directly affecting the integrity of the K88 pili or by other membrane related mechanisms is unknown at present. Furthermore, the presence of PMBN has been found to reduce the frequency of conjugal gene transfer in E.coli (A.Matranga, P.Bennett & I.Chopra, personal communication). This finding could be the consequence of interference with cell to cell contact by PMBN. Therefore despite the disappointing observations made by Lam et al., 1986, PMBN may nevertheless prove to have future

applications in human or veterinary medicine.

REFERENCES

REFERENCES

ALATOSSAVA,T.,VAARA,M.& BASCHONG,W.(1984). Polymyxin B Nonapeptide sensitizes E.coli to Valinomycin and A23187 ionophores. FEMS Microbiology Letters 22:249-251.

AMES,G.F-L.& NIKAIDO,H.(1976). Two-dimensional gel electrophoresis of membrane proteins. Biochemistry 15:616-623.

ANWAR,H.,BROWN,M.R.W.,DAY,A.& WELLER,P.H.(1984). Outer membrane antigens of mucoid Pseudomonas aeruginosa isolated directly from the sputum of a cystic fibrosis patient. FEMS Microbiology letters 24:235-239.

BIRD,A.E.,CHARSLEY C-H.,JENNINGS,K.R.& MARSHALL,A.C.(1984). High performance liquid chromatographic assay of Temocillin and epimerisation of its diastereoisomers. Analyst 109:1209-1212.

BOYER,H.W.,BETLACH,M.,BOLIVAR,F.,RODRIGUEZ,R.L.,HEYNEKER,H.L., SHINE,J.& GOODMAN,H.M.(1977). The construction of molecular cloning vehicles. In Recombinant molecules: Impact on science and society. (Beers,R.F.& Bassett,E.G.,Eds.) pp.9-20. Raven Press, New York.

BRADMAN,M.M.(1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. Analytical Biochemistry

72:248-254.

BRASS,J.M.,HIGGINS,C.F.,FOLEY,M.,RUGMAN,P.A.,BIRMINGHAM, J.& GARLAND,P.B.(1986). Lateral diffusion of proteins in the periplasm of Escherichia coli. Journal of Bacteriology 165:787-794.

BRAUN,V.& HANTKE,K.(1974). Biochemistry of bacterial cell envelopes. Annual Review of Biochemistry 43:89-121.

BRITTEN,R.J.& McCLURE,F.T.(1962). The amino acid pool in Escherichia coli. Bacteriological Reviews 26: 292-335.

BROWN,M.R.W.(1975). The role of the cell envelope in resistance. In Resistance of Pseudomonas aeruginosa (Brown,M.R.W.Ed.). pp.71-107. John Wiley and Sons, London.

BUKHARI,A.L.,SHAPIRO,J.L.& ADHYA,S.L.(1977). DNA insertion elements, plasmids and episomes. Cold Spring Harbor Laboratory, Cold Spring Harbor.

CARR,C.Jnr.& MORRISON,D.C.(1985). Mechanism of polymyxin B-mediated lysis of lipopolysaccharide-treated erythrocytes. Infection and Immunity 49:84-89.

CERNY,G.& TEUBER,M.(1971). Differential release of periplasmic versus cytoplasmic enzymes from Escherichia coli by polymyxin B. Archives of Microbiology 78:166-179.

CERNY,G.& TEUBER,M.(1972). Comparative polyacrylamide electrophoresis of periplasmic proteins released from Gram-negative bacteria by polymyxin B. Archives of Microbiology 82:361-370.

CHAMBERLAIN,J.P.(1979). Fluorographic detection of radio-activity in polyacrylamide gels with the water soluble fluor, sodium salicylate. Analytical Biochemistry 98:132-135.

CHAPMAN,G.B.(1962). Cytological aspects of antimicrobial antibiosis. I Cytological changes associated with the exposure of Escherichia coli to colistin sulfate. Journal of Bacteriology 84:169-179.

CHEN,R.,SCHMIDMAYR,W.,KRAMER,C.,CHEN-SCHMEISSER,U.& HENNING,U.(1980). Primary structure of major outer membrane protein II (OmpA protein) of Escherichia coli K12. Proceedings of National Academy of Sciences U.S.A. 77:4592-4596.

CHEN,W.C.,CHOU,D.L.& FEINGOLD,D.S.(1978). Dissociation between ion permeability and lethal action of polyene antibiotics on Candida albicans. Antimicrobial Agents and Chemotherapy 13:914-917.

CHIHARA,S.,TOBITA,T.,YAHATA,M.,ITO,A.& KOYAMA,Y.(1973). Enzymatic degradation of colistin isolation and identification

of a-N-Acyl a,-Diaminobutyric acid and colistin nonapeptide. Agricultural and Biological Chemistry 37:2455-2463.

CHIHARA,S.,ITO,A.,YAHATA,M.,TOBITA,T.& KOYAMA,Y.(1974). Chemical synthesis, isolation and characterization of a-N-Fattyacyl colistin nonapeptide with special reference to the correlation between antimicrobial activity and carbon number of fattyacyl moiety. Agricultural and Biological Chemistry 38:521-529.

CHOPRA,I.,HOWE,T.G.B.& BALL,P.R.(1977). Lysozyme-promoted association of protein I molecules in the outer membrane of Escherichia coli. Journal of Bacteriology 132:411-418.

CHOPRA,I.& ECCLES,S.J.(1978). Diffusion of tetracycline across the outer membrane of Escherichia coli K12: Involvement of protein Ia. Biochemical and Biophysical Research Communications 83:550-557.

CHOPRA,I.& SHALES,S.W.(1980). Comparison of the polypeptide composition of Escherichia coli outer membranes prepared by two methods. Journal of Bacteriology 144:425-427.

CHOPRA,I.(1985a). Mode of action of the tetracyclines and the nature of bacterial resistance to them. In Handbook of experimental pharmacology, Vol.78 (Hlavka,J.J.& Boothe,J.H.,Eds.).pp.317-392. Springer-Verlag.

CHOPRA,I.(1985b). Using a microcomputer with a spectrophotometer. Laboratory Practice pp 21-22 September 1985.

CHOPRA,I.& LINTON,A.H.(1986). The antibacterial effects of low concentrations of antibiotics. Advances in Microbial Physiology 28:211-260.

CHOPRA,I.(1986). Antibiotics and bacterial adhesion. Journal of Antimicrobial Chemotherapy 18:553-554.

COSTERTON,J.W.,INGRAM,J.M.& CHENG,K.J.(1974). Structure and function of the cell envelope of Gram-negative bacteria. Bacteriological Reviews 38:87-110.

COUGHLIN,R.T.,CALDWELL,C.R.,HAUG,A.& McGROARTY,E.J.(1981). A cationic electron spin resonance probe used to analyze cation interactions with lipopolysaccharide. Biochemical and Biophysical Research Communications 100:1137-1142.

CURTIS,N.A.C.,BROWN,C.,BOXALL,M.& BOULTON,M.G.(1979a). Inhibition of Escherichia coli K12 by beta-lactam antibiotics with poor antibacterial activity: Interaction of permeability and intrinsic activity against penicillin-binding proteins. Antimicrobial Agents and Chemotherapy 15:332-336.

CURTIS,N.A.C.,ORR,D.,ROSS,G.W.& BOULTON,M.G.(1979b). Affinities of penicillins and cephalosporins for the penicillin-binding proteins of Escherichia coli K12 and their antibacterial

activity. Antimicrobial Agents and Chemotherapy 16:533-539.

CURTISS,R.(1981). Gene transfer. In Manual of methods for general bacteriology (Gerhardt,P.Ed.) pp 243-265. American Society for Microbiology, Washington.

DEPAMPHILIS,M.L.(1971). Dissociation and reassembly of Escherichia coli outer membrane and of lipopolysaccharide and their reassembly onto flagellar basal bodies. Journal of Bacteriology 105:1184-1199.

DIRIENZO,J.M.,NAKAMURA,K.& INOUYE,M.(1978). The outer membrane proteins of Gram-negative bacteria: biosynthesis, assembly and functions. Annual Review of Biochemistry 47:481-532.

DOUGHERTY,T.J.& SAUKKONEN,J.J.(1985). Membrane permeability changes associated with DNA gyrase inhibitors in Escherichia coli. Antimicrobial Agents and Chemotherapy 28: 200-206.

EL MASHAK,E.M.& TOCANNE,J.F.(1980). Polymyxin B phosphatidyl -glyerol interactions. Biochimica.et Biophysica acta 596:165-179.

ENGELHARDT,H.(1979). High performance liquid chromatography. Springer-Verlag.

FILIP,C.,FLETCHER,G.,WULFF,J.L.& EARHART,C.F.(1973). Solubilization of the cytoplasmic membrane of Escherichia coli by the ionic detergent sodium-lauryl sarcosinate. Journal of Bacteriology 115:717-722.

GAASTRA,W.& De GRAAF,F.K.(1982). Host specific fimbrial adhesions of non-invasive enterotoxigenic Escherichia coli strains. Microbiological Reviews 46:129-161.

GALANOS,C.,LUDERITZ,O.,RIETSCHEL,E.T.& WESTPHAL,O.(1977). Newer aspects of the chemistry and biology of bacterial polysaccharides with special reference to their lipid A component In International review of Biochemistry: Biochemistry of lipids II (Goodwin,T.W.Ed.) 14:239-335 University Park Press, Baltimore.

GALE,E.F.,CUNDLIFFE,E.,REYNOLDS,P.E.,RICHMOND,M.H.& WARING,M.J.(1981). The molecular basis of antibiotic action. John Wiley & Sons, London.

GILLELAND,H.E.Jnr.& MURRAY,R.G.E.(1976). Ultrastructural study of polymyxin-resistant isolates of Pseudomonas aeruginosa. Journal of Bacteriology 125:267-281.

GUSTAFSSON,P.,NORDSTROM,K.& NORMARK,S.(1973). Outer penetration barrier of Escherichia coli K12: kinetics of the uptake of gentian violet by wild type and envelope mutants. Journal of Bacteriology 116:893-900.

HALL,M.N.& SILHAVY,T.J.(1981). Genetic analysis of the major outer membrane proteins of Escherichia coli. Annual Review of Genetics 15:91-142.

HALLEWELL,R.A.& SHERRATT,D.J.(1976). Isolation and characterization of ColE2 plasmid mutants unable to kill colicin-sensitive cells. Molecular and General Genetics 146:239-245.

HAMES,B.D.(1981). An introduction to polyacrylamide gel electrophoresis. In Gel electrophoresis of proteins: a practical approach. (Hames,B.D.& Rickwood,D.,Eds.). pp.1-91. I.R.L.Press Ltd.,London.

HAMMOND,S.M.,LAMBERT,P.A.& RYCROFT,A.N.(1984). The bacterial cell surface. Croom Helm, Beckenham, Kent.

HANCOCK,R.E.W.(1981). Aminoglycoside uptake and mode of action - with special reference to streptomycin and gentamicin II. Effects of amino-glycosides on cells. Journal of Antimicrobial Chemotherapy 8:429-445.

HANCOCK,R.E.W.(1984). Alterations in outer membrane permeability. Annual Review of Microbiology 38:237-64.

HARTMANN,R.,BOCK-HENNING,S.B.& SCHWARTZ,U.(1974). Murein hydrolase in the envelope of Escherichia coli. Properties in

situ and solubilization from the envelope. European Journal of Biochemistry 41:203-208.

HARTMANN,W.,GALLA,H-J.& SACKMANN,E.(1978). Polymyxin binding to charged lipid membranes an example of cooperative lipid-protein interaction. Biochimica et Biophysica Acta 510:124-139.

HEDGES,R.W.,RODRIGUEZ-LEMOINE,V.& DATTA,N.(1975). R-factors from Serratia marcescens. Journal of General Microbiology 86:88-92.

HENNING,U.,SONNTAG,I.& HINDENNACH,I.(1978). Mutants (OmpA) affecting a major outer membrane protein of Escherichia coli K12. European Journal of Biochemistry 92:491-498.

HEPPEL,L.A.(1968). Preparation of cells of Escherichia coli with altered permeability. Methods in Enzymology 12 part B:841-846.

HOBOT,J.A.,CARLEMALM,E.,VILLIGER,W.& KELLENBERGER,E.(1984). Periplasmic gel: New concept resulting from the reinvestigation of bacterial cell envelope ultrastructure by new methods. Journal of Bacteriology 160:143-152.

HOLT,S.C.& BEVERIDGE,T.J.(1982). Electron microscopy: its development and application to microbiology. Canadian Journal of Microbiology 28:1-52

HOLTJE,J-V.& SCHWARZ,U.(1985). Biosynthesis and growth of the murein sacculus. In Molecular Cytology of Escherichia coli (Nanninga,N.,Ed.) pp177-219. Academic Press,London.

HSU-CHEN,C.C.& FEINGOLD,D.S.(1973). The mechanism of polymyxin B action selectively towards biological membranes. Biochemistry 12:2105-2111.

HUGO,W.B.(1967). The mode of action of antibacterial agents. Journal of Applied Bacteriology.30:17-45.

HUGO,W.B.(1982). Disinfection mechanisms. In Principles and practice of disinfection, preservation and sterilisation (Russell,A.D.,Hugo,W.B.& Ayliffe,G.A.J.Eds). p158. Blackwell Scientific Publications, Oxford.

HUMPHREYS,G.O.,WILLSHAW,G.A.& ANDERSON,E.(1975). A simple method for the preparation of large quantities of pure plasmid DNA. Biochimica et Biophysica Acta 383:457-463.

ICHIHARA,S.& MIZUSHIMA,S.(1978). Characterization of major outer membrane proteins 0-8 and 0-9 of Escherichia coli K12. Journal of Biochemistry 83:1095-1100.

INGRAHAM,J.L.,MAALOE,O.& NEIDHARDT,F.C.(1983). Growth of the bacterial cell. Sinauer Associates Ltd.

JACOBSON,G.R.,TAKACS,B.J.& ROSENBUSCH,J.P.(1976). Properties of

a major protein released from Escherichia coli by osmotic shock. Biochemistry 15:2297-2303.

KARKHANIS,Y.D.,ZELTNER,J.Y.,JACKSON,J.J.& CARLO,D.J.(1978). A new and improved microassay to determine 2-keto-3-deoxyoctonate in the lipopolysaccharide of Gram-negative bacteria. Analytical Biochemistry 85:595-599.

KATSU,T.,YOSHIMURA,S.& FUJITA,Y.(1984a). Increases in permeability of Escherichia coli outer membrane induced by polycations. FEBS Letters 166:175-178.

KATSU,T.,YOSHIMURA,S.,TSUCHIDA,T.& FUJITA,Y.(1984b). Temperature dependence of action of polymyxin B on Escherichia coli. Journal of Biochemistry 95: 1645-1653.

KLEMPERER,R.M.,GILBERT,P.,MEIER,A.M.,COZENS,R.M.& BROWN,M.R.W.(1979). Influence of suspending media upon the susceptibility of Pseudomonas aeruginosa NCTC 6750 and its spheroplasts to polymyxin B. Antimicrobial Agents and Chemotherapy 15:147-151.

KOIKE,M.,IIDA,K.& MATSUO,T.(1969). Electron microscopic studies on mode of action of polymyxin. Journal of Bacteriology 97:443-452.

KOIKE,M.& IIDA,K.(1971). Effect of polymyxin on the

bacteriophage receptors of the cell walls of Gram-negative bacteria. Journal of Bacteriology 108:1402-1411.

KOPYLOVA-SVIRIDOVA,T.N.,SOUKOVATITSIN,V.V.& FODOR,I.(1979). Synthesis of proteins coded by plasmid vectors of pcv series (Ap^R,Tc^R) and their recombinant derivatives (pDm) in Escherichia coli minicells. Gene 7:121-139.

KROGSTAD,D.J.& MOELLERING,R.C.(1980). Combinations of antibiotics, mechanisms of interaction against bacteria. In Antibiotics in laboratory medicine (Lorian,V.Ed.) pp.298-341. The Williams and Wilkins Co.,Baltimore.

LABISCHINSKI,H.,BARNICKEL,G.,BRADACZEK,H.,NAUMANN,D., RIETSCHEL,E.T.& GIESBRECHT,P.(1985). High state of order of isolated bacterial lipopolysaccharide and its possible contribution to the permeation barrier property of the outer membrane. Journal of Bacteriology 162:9-20.

LAEMMLI,U.K.(1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

LAM,C.,HILDEBRANDT,J.,SCHUTZE,E.& WENZEL,A.F.(1986). Membrane -disorganizing property of polymyxin B nonapeptide. Journal of ntimicrobial Chemotherapy 18:9-15.

LANNIGAN,R.& BRYAN,L.E.(1985). Decreased susceptibility of

Serratia marcescens to chlorhexidine related to the inner membrane. Journal of Antimicrobial Chemotherapy 15: 559-565.

LEIVE,L.,SHOVLIN,V.K.& MERHAGEN,S.E.(1968). Physical, chemical and immunological properties of lipopolysaccharide release from Escherichia coli by ethylenediaminetetraacetate. Journal of Biological Chemistry 243:6384-6491.

LEIVE,L.(1974). The barrier function of Gram-negative envelope. Annals of New York Academy of Sciences 235:109-129.

LOUNATMAA,K.,MAKELA,P.H.& SARVAS,M.(1976). Effect of polymyxin on the ultrastructure of the outer membrane of wild-type and polymyxin-resistant strains of Salmonella. Journal of Bacteriology 127:1400-1407.

LUDERITZ,O.,FREUDENBERG,M.A.,GALANOS,C.,LEHMANN,V.,REITSCHEL, E.T.& SHAW,D.H.(1982). Lipopolysaccharides of Gram-negative bacteria. Current Topics in Membrane Transport 17:79-151.

LUGTENBERG,B.& VAN ALPHEN,L.(1983). Molecular architecture and functioning of the outer membrane of Escherichia coli and other Gram-negative bacteria. Biochimica et Biophysica Acta 737:51-115.

LUGTENBERG,E.J.J.& PETERS,R.(1976). Distribution of lipids in cytoplasmic and outer membranes of Escherichia coli K12.

Biochimica et Biophysica Acta 441:38-47.

LURIA,S.E.,ADAMS,J.N.& TING,R.C.(1960). Transduction of lactose-utilizing ability among strains of Escherichia coli and Shigella dysenteriae and the properties of the transducing phage particles. Virology 12:348-390.

M CORMICK,J.R.D.,FOX,S.M.,SMITH,L.L.,BITLER,B.A., REICHENTHAL,J.,ORIGONI,V.E.,MULLER,W.H.,WINTERBOTTOM,R.& DOERSCHUK,A.P.(1957). Studies of the reversible epimerization occurring in the tetracycline family. The preparation, properties and proof of structure of some 4-epi-tetracyclines. Journal of American Chemical Society 79:2849-2859.

MARTIN,N.L.& BEVERIDGE,T.J.(1986). Gentamicin interaction with Pseudomonas aeruginosa cell envelope. Antimicrobial Agents and Chemotherapy 29:1079-1087.

MILES,A.A.,MISRA,S.S.& IRWIN,J.O.(1938). The estimation of the bacteriocidal power of the blood. Journal of Hygiene 38: 732.

MORRISON,D.C.& JACOBS,D.M.(1976). Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochemistry 13:813-818.

NEU,H.& HEPPEL,L.A.(1965). The release of enzymes from Escherichia coli by osmotic shock and during the formation of

spheroplasts. The Journal of Biological Chemistry 240: 3685-3692.

NEWTON,B.A.(1953). Reversal of the antimicrobial activity of polymyxin by divalent cations. Nature (London) 172:160-161.

NEWTON,B.A.(1954). Site of action of polymyxin on Pseudomonas aeruginosa: Antagonism by cations. Journal of General Microbiology 10:491-499.

NEWTON,B.A.(1956). The properties and mode of action of the polymyxins. Bacteriological Reviews 20:14-27.

NICAS,T.I.& HANCOCK,R.E.W.(1980). Outer membrane protein H1 of Pseudomonas aeruginosa: Involvement in adaptive and mutational resistance to ethylenediaminetetraacetate, polymyxin B and gentamicin. Journal of Bacteriology 143:872-878.

NIKAIDO,H.(1973). Biosynthesis and assembly of lipo-polysaccharide and the outer membrane layer of Gram-negative cell wall. In Bacterial membranes and walls. (Leive,L.Ed.) pp.131-208. Marcel Dekker, New York.

NIKAIDO,H.(1976). Outer membrane of Salmonella typhimurium: transmembrane diffusion of some hydrophobic substances. Biochimica et Biophysica Acta 433:118-132.

NIKAIDO,H.(1985). Role of permeability barriers in resistance to beta-lactam antibiotics. Pharmacology and Therapeutics 27:197-231.

NIKAIDO,H.& NAKAE,T.(1979). The outer membrane of Gram-negative bacteria. Advances in Microbial Physiology 20:163-250.

NIKAIDO,H.& VAARA,M.(1985). Molecular basis of bacterial outer membrane permeability. Microbiological Reviews 49:1-32.

O'CALLAGHAN,C.H.,MORRIS,A.,KIRBY,S.M.& SHINGLER,A.H.(1972). Novel method for the detection of B-lactamases by using a chromogenic cephalosporin substrate. Antimicrobial Agents and Chemotherapy 1:283-288.

O'FARRELL,P.H.(1975). High resolution two dimensional electrophoresis of proteins. Journal of Biological Chemistry 250:4007-4021.

ORSKOV,I.,ORSKOV,F.,JANN,B.& JANN,K.(1977). Serology, chemistry and genetics of O and K antigens of Escherichia coli. Bacteriological Reviews 41:667-710.

OSBORN,M.J.,GANDER,J.E.,PARISI,E.& CARSON,J.(1972). Mechanism of assembly of the outer membrane of Salmonella typhimurium. I. Isolation and characterization of cytoplasmic and outer membranes. Journal of Biological Chemistry 247:3962-3972.

OSBORN,M.J.& WU,H.C.P.(1980). Proteins of the outer membrane of Gram-negative bacteria. Annual Review of Microbiology **34**:369-422.

OVERBEEKE,N.,van SCHARRENBURG,G.& LUGTENBERG,B.(1980). Antigenic relationships between pore proteins of Escherichia coli K12. European Journal of Biochemistry **110**:247-254.

PETERSON,A.A.,HANCOCK,R.E.W.& McGROARTY,E.J.(1985). Binding of polycationic antibiotics and polyamines to lipopolysaccharides of Pseudomonas aeruginosa. Journal of Bacteriology **164**: 1256-1261.

PLESTED,S.J.,SIMPSON,I.N.& JAMES,M.(1983). The detection of bacterial B-lactamases and their identification by analytical isoelectric focusing. In Antibiotics: Assessment of antimicrobial activity and resistance. (Russell,A.D.& Quesnel,L.B.,Eds.) pp.111-126. Technical series of the Society for Applied Bacteriology, Vol.18. Academic Press, London.

PREHM,P.,STIRM,S.,JANN,B.& JANN,K.(1976). Cell-wall lipopolysaccharides of ampicillin-resistant mutants of Escherichia coli K12. European Journal of Biochemistry **66**:369-377.

RICHMOND,M.H.& WOTTON,S.(1976). Comparative study of seven cephalosporins: susceptibility to beta-lactamases and ability to penetrate the surface layers of Escherichia coli.

Antimicrobial Agents and Chemotherapy 10:219-222.

RICHMOND,M.H.& SYKES,R.B.(1973). The B-lactamases of Gram-negative bacteria and their possible physiological role. In Advances in Microbial Physiology, Vol.9.(Rose,A.H.& Tempest,D.W.,Eds.). pp.31-88. Academic Press Inc., New York.

ROBERTS,J.K.M.& JARDETZKY,O.(1985) Nuclear magnetic resonance spectroscopy in biochemistry. In Modern physical methods in biochemistry, part A (Neuberger,A and Van Deenan,L.L.M., Eds.). pp.1-67. Elsevier, Amsterdam.

ROGERS,H.J.,PERKINS,H.R.& WARD,J.B.(1980). Microbial cell walls and membranes. Chapman and Hall, London.

ROSENTHAL,K.S.,SWANSON,P.E.& STORM,D.R.(1976). Disruption of Escherichia coli outer membranes by EM49. A new membrane active peptide antibiotic. Biochemistry 15:5783-5792.

ROSS,G.W.& O'CALLAGHAN,C.H.(1975). B-lactamase assays. In Antibiotics, Methods in Enzymology, Vol.43 (Hash,J.H.,Ed.) pp.69-85. Academic Press Inc., New York.

SALTON,M.R.J.& OWEN,P.(1976). Bacterial membrane structure. Annual Review of Microbiology 30:451-482.

SCHINDLER,P.R.G.& TEUBER,M.(1975). Action of polymyxin B on bacterial membranes: morphological changes in the cytoplasm and

in the outer membrane of Salmonella typhimurium and Escherichia coli B. Antimicrobial Agents and Chemotherapy 8:95-104.

SCHINDLER,M.& OSBORN,M.J.(1979). Interaction of divalent cations and polymyxin B with lipopolysaccharide. Biochemistry 18:4425-4430.

SCHLEIFER,K.H.& KANDLER,O.(1972). Peptidoglycan types of bacterial cell walls and their taxonomic implications. Bacteriological Reviews 36:407-477.

SHALES,S.W.& CHOPRA,I.(1982). Outer membrane composition in Escherichia coli and the poor activity of hydrophobic antibiotics against enteric bacteria. Journal of Antimicrobial Chemotherapy 9:325-331.

SHERMA,J.& FRIED,B.(1984). Flat bed techniques. In Separation methods (Deyl,Z.Ed.) pp.363-413. Elsevier, Amsterdam.

SIXL,F.& GALLA,H.J.(1982). Calorimetric investigation of polymyxin binding to phosphatidic acid bilayers. Biochimica et Biophysica Acta 693:466-478.

SMYTH,C.J.(1985). Immunology of outer membrane proteins of Gram-negative bacteria. In Immunology of the bacterial cell envelope (Stewart-Tull,D.E.S.& Davies,M.Eds.) John Wiley and Sons Ltd.

SPRATT,B.G.(1977). Properties of the penicillin-binding proteins of Escherichia coli K12. European Journal of Biochemistry 72:341-352.

STORM,D.R.,ROSENTHAL,K.S.& SWANSON,P.E.(1977). Polymyxin and related peptide antibiotics. Annual Reviews of Biochemistry 46:723-763.

SUGANUMA,S.,HARA,K.,KISHIDA,T.,NAKAJIMA,K.& KAWAMATA,J.(1968). Cytological changes of Escherichia coli caused by polymyxin E. Biken J. 11:149-155.

SUZUKI,H.,NISHIMURA,Y.,YASUDA,S.,NISHIMURA,A.,YAMADA,M.& HIROTA,Y.(1978). Murein-lipoprotein of Escherichia coli: a protein involved in the stabilization of bacterial cell envelope. Molecular and General Genetics 167:1-9.

TAI,T.Y.& VAN HEYNINGEN,W.E.(1951). Bacteriolysis by a species of Streptomyces. Journal of General Microbiology 5:110-120.

TEUBER,M.(1970). Lysozyme-dependent production of spheroplast-like bodies from polymyxin B-treated Salmonella typhimurium. Archives of Microbiology 70:139-146.

TEUBER,M.(1974). Action of polymyxin B on bacterial membranes. III Differential inhibition of cellular function in Salmonella

typhimurium. Archives of Microbiology 100:131-144.

TEUBER,M.& BADER,J.(1976). Action of polymyxin on bacterial membranes. Binding capacities for polymyxin B of inner and outer membranes isolated from Salmonella typhimurium G30. Archives of Microbiology 109:51-58.

THERETZ,A.,TEISSIE,J.& TOCANNE,J-F.(1984). A study of the structure and dynamics of complexes between polymyxin B and phosphatidylglycerol in monolayers by fluorescence. European Journal of Biochemistry 142:113-119.

TOWBIN,H.,STAEHELIN,T.& GORDON,J.(1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of the National Academy of Sciences 76:4350-4354.

TSUCHIDO,T.,KATSUI,N.,TAKEUCH,A.,TAKANO,M.& SHIBASAKI,I. (1985). Destruction of the outer membrane permeability barrier of Escherichia coli by heat treatment. Applied and Environmental Microbiology 50:298-303.

VAARA,M.,VAARA,T.& SARVAS,M.(1979). Decreased binding of polymyxin by polymyxin-resistant mutants of Salmonella typhimurium. Journal of Bacteriology 139:664-667.

VAARA,M.(1983). Polymyxin B Nonapeptide complexes with lipopolysaccharide. FEMS Microbiology Letters 18:117-121.

VAARA,M.& VAARA,T.(1981). Outer membrane permeability barrier disruption by polymyxin in polymyxin-susceptible and -resistant Salmonella typhimurium. Antimicrobial Agents and Chemotherapy 19:578-583.

VAARA,M.& VAARA,T.(1983a). Polycations sensitize enteric bacteria to antibiotics. Antimicrobial Agents and Chemotherapy 24:107-113.

VAARA,M.& VAARA,T.(1983b). Polycations as outer membrane disorganizing agents. Antimicrobial Agents and Chemotherapy. 24:114-122.

VAARA,M.& VAARA,T.(1983c). Sensitization of Gram-negative bacteria to antibiotics and complememt by a non-toxic oligopeptide. Nature 303:526-528.

VAARA,M.& VILJANEN,P.(1983). Outer membrane phospholipase is not the mediator in the bactericidal or outer membrane permeability-increasing action of polycations. FEMS Microbiology Letters 19:253-256.

VAARA,M.& VILJANEN,P.(1985). Binding of Polymyxin B Nonapeptide to Gram-negative bacteria. Antimicrobial Agents and Chemotherapy 27:548-555.

VAARA,M.,VILJANEN,P.,SUKUPOLVI,S.& VAARA,T.(1985). Does

Polymyxin B Nonapeptide increase outer membrane permeability in antibiotic supersensitive enterobacterial mutants? FEMS Microbiological Letters 26:289-294.

VILJANEN,P.& VAARA,M.(1984). Susceptibility of Gram-negative bacteria to Polymyxin B Nonapeptide. Antimicrobial Agents and Chemotherapy 25:701-705.

WAHN,K.,LUTSCH,G.,ROCKSTROH,T.& ZAPT,K.(1968). Morphological and physiological investigations on the action of polymyxin B on Escherichia coli. Archives of Microbiology 63: 103-116

WHITE,H.J.,ALVERSON,C.M.,BAKER,M.J.& BAKER,E.R.(1949). Comparative biological studies of polymyxins and "aerosporin". Annals of the New York Academy of Sciences 51:879-890.

WILKINSON,S.G.(1975). Sensitivity to ethylenediamine tetraacetic acid. In Resistance to Pseudomonas aeruginosa. (Brown,M.R.W.Ed.) pp 145-188. John Wiley and Sons, London.

WILLIS,R.C.,MORRIS,R.G.,CIRAKOGLU,C.,SCHELLENBERG,G.D., GERBER,N.H.& FURLONG,C.E.(1974). Preparation of the periplasmic binding proteins from Salmonella typhimurium and Escherichia coli. Archives of Biochemistry and Biophysics 161:64-75.

YAMATO,I.,ANRAKU,Y.& HIROSAWA,K.(1975). Cytoplasmic membrane vesicles of Escherichia coli. I. A simple method of preparing

the cytoplasmic and outer membranes. Journal of Biochemistry 77:705-718.

ZIMMERMAN,W.& ROSSELET,A.(1977). Function of the outer membrane of Escherichia coli as a permeability barrier to beta-lactam antibiotics. Antimicrobial Agents and Chemotherapy 12: 368-372.